

**Studies into the insecticidal activity and mode of action  
of monoterpenoid constituents of essential oils against  
the human louse, *Pediculus humanus*.**

A thesis presented by

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## Abstract

The incidence of head lice, *Pediculus humanus capitis*, in the West is increasing, with insecticide resistance the likely cause. Previous studies have explored the utility of essential oils, and some of their constituent monoterpenoids, in the treatment of head lice. This investigation examines the relative short-term toxicity of a range of different monoterpenoid structures on adult clothing lice, *Pediculus humanus corporis*, and their eggs; a structure-activity series was generated for the adults, and partially for eggs. The most effective monoterpenoid against adult lice was (+)-terpinen-4-ol, with monocyclic compounds containing a single O-atom having the highest activities. Furthermore, there appear to be important differences between the relative potencies of monoterpenoids on lice and eggs, as nerolidol was particularly effective against eggs but completely ineffective against adult lice.

To investigate the insecticidal mechanism of action of monoterpenoids, various pediculicidal structures were screened for activity on an insect ionotropic GABA receptor, composed of the *Drosophila melanogaster* subunit RDL<sub>ac</sub>, expressed in *Xenopus* oocytes. Thymol, eugenol and carvacrol potentiated GABA responses at this receptor, and possessed agonist activity at high concentrations. This is the first documentation of monoterpenoid bioactivity at an isolated insect receptor known to be representative of an *in vivo* insecticidal target.

Thymol also had potentiating and agonist effects on human  $\alpha 1\beta 3\gamma 2$ s GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes, and 50  $\mu$ M thymol induced a leftwards shift of the GABA dose-response curve. Further work on this receptor examined the interaction of thymol with previously characterised modulator binding sites. The results of functional studies suggest that thymol does not share a binding site with benzodiazepines, barbiturates, steroids, propofol,  $\beta$ -carbolines or loreclezole.

The direct involvement of insect GABA receptors in monoterpenoid insecticidal activity remains to be confirmed, as does the location of the thymol binding site on insect and mammalian ionotropic GABA receptors.

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# **Chapter 1**

## **Introduction**

### **1.1 Concepts of head louse control**

Lice have been known to parasitise humans for thousands of years. Remains of lice have even been found on Egyptian mummies approximately 5000 years old [11]. Historically, lice were associated with disease and dirt, no doubt because the clothing louse of man was responsible for the spread of terrible illnesses such as typhus and trench fever. This association parallels the infamy of the black rat in being a plague-carrier. In the same way that the reputation of the brown rat, which is not a plague vector, was harmed by its association with the black rat, so the reputation of the head louse was harmed by association with its close relative, the clothing louse.

Social attitudes to head lice infection vary greatly between societies, and even within different segments of a community. Westerners are generally embarrassed to admit they have lice whereas in poorer parts of the world they have always been viewed as a fact of life [81]. In the 20<sup>th</sup> Century, the gradual introduction of pediculicides (agents lethal to lice) and concomitant gradual decrease in tolerance of lice led to falling infestation rates in Western societies. In the U.K., the incidence of head lice was probably at its lowest in the late 1980s and early 1990s, but since then resistance has destroyed the usefulness of the majority of approved pharmaceutical interventions.

Unfortunately, Western OTC (over the counter) and POM (prescription only medicine) pediculicides are expensive. The cost of treating head lice was estimated at 367 million dollars per annum in the USA in the early 1990s [69]. In developing countries, where the extent of the problem is far greater, such treatments are either unavailable or prohibitively expensive [81]. Cures for pediculosis (an infestation of human lice) continue to centre around social grooming and ancient remedies, many of which are based on natural products. However, little would be known of the efficacy, selectivity and side-effects of such remedies.

Since around 1991, there has been increasing anecdotal evidence from health professionals and parents alike supporting a recrudescence of head lice in the West [51]. The sudden

widespread occurrence and the desperation experienced by parents in the face of treatment failures have perhaps precipitated the dissipation of the taboo surrounding the discussion of lice, resulting in a large number of media articles on louse control.

There are a great many plants with reputed anti-lice activity [128] and therefore the scientific evaluation of natural products, perhaps from popular remedies of the developing world, could provide new avenues in louse control.

## **1.2 The taxonomy and anatomy of human lice and their interrelationship**

Lice are said to be the most fully parasitic of all insects, as every stage of their life-cycle is intimately associated with the host. As a result of the permanent ectoparasitic state, all lice are highly specialised. Lice species are extremely host-specific, colonising one or a small number of animal species. They are often further adapted to a particular anatomical part, and therefore multiple louse species may infest a single host without competition [160].

There are three orders of lice, according to Maunder [160]. The Mallophaga or 'chewing lice' are skin eaters, this order contains 2,600 species and most are parasites of birds. The Rhyncophthiraptera, 'cutting lice,' contains two species, one parasitising elephants, the other warthogs. The Anoplura comprises over 565 species of sucking lice [160,62,84] and are found on nearly all groups of mammals [45]. Within the Order Anoplura are two Families of lice that encompass the three types of louse colonising humans [134]. Each type infests a different area of the body. The nomenclature adopted here is that of Maunder [160]: The head louse, *Pediculus humanus* (Linnaeus) *capitis* and the body, or clothing, louse, *Pediculus humanus corporis*, belong to the family Pediculidae. Collectively, *P. h. capitis* and *P. h. corporis* are referred to as *P. humanus*. The pubic, or crab, louse, *Phthirus pubis* belongs to Pthiridae. In Western Society at present, the head louse is of greatest clinical concern, therefore louse-control measures and associated mechanistic aspects discussed here will focus on *P. h. capitis*, although most louse-control methods are applicable to all three types.

Obligate parasites, such as *P. humanus* and *P. pubis*, characteristically occupy very narrow, fragile biological niches [205]. During evolution, it is almost as if characteristics which enabled them to survive in other habitats were exchanged for those which would allow them the best chance of survival in their precise yet precarious environment. Visible behavioural and morphological traits of lice clearly display the extremity of this adaptation.

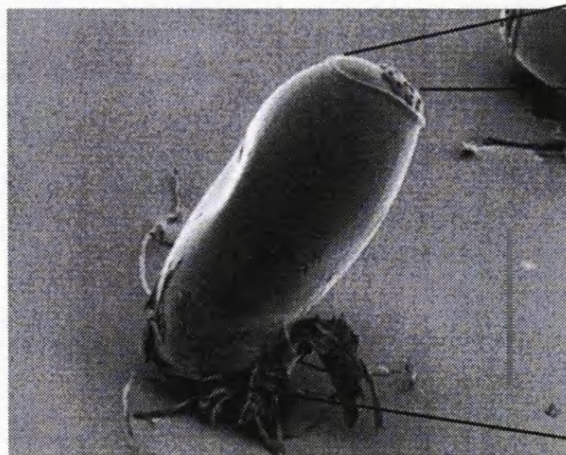
For example, neither head lice nor their eggs are able to survive distal to the host for more than 1-2 days unless provided with a climate of artificially enhanced temperature and humidity [205]. All human lice are obligate haematophages and carry symbiotic microorganisms to supplement nutrition. They have no wings and much reduced sensory function in comparison to even temporary parasites, yet the consequentially short antennae, along with the ability to retract their mouthparts, helps to minimise the potential for damage via fragile areas [148]. Spines and bristles on the cuticle probably help to protect delicate areas of the cuticle from abrasion by hairs. Adult lice are small, and all life-cycle stages are camouflaged, which reduces the chances of host-detection. The tarsal claws are modified for gripping which aids movement through fibrous media, as does their dorsoventral flattening. The latter also allows adpression to scalp and this, along with the capacity to grip incredibly firmly, helps lice to remain attached to their medium during host grooming activities [148].

The three varieties of human lice have discrete, non-overlapping distributions on the body, hence the common nomenclature. Some of the adaptations to these three different microclimates are also clearly visible, for example, the forelimbs of *P. pubis* are more widely spaced than those of *P. humanus*, just as pubic hair follicles are farther apart than those of body hair. However, *P. h. capitis* and *P. h. corporis* are not easy to differentiate; there are very slight but consistent morphological differences between the two groups of organisms yet there is also overlap between the sub-species in respect of these features [57,58]. Busvine suggested that a physical separation of *P. humanus* was followed by continuing adaptation of the two sub-populations according to the specific habitat conditions, such as food availability and environmental stability, therefore head and clothing lice were two separate species. However, head and clothing lice freely interbreed *in vitro* and, given that the two habitats are proximal *in vivo*, probably also do so in the wild [57]. For this reason they have been designated sub-species, rather than species, classification [160]. Even under the electron microscope, head lice and clothing lice appear identical without morphometric assessment. Figure 1.1 shows scanning electron micrographs (SEMs) of *P. humanus* adult and egg.



(a)

500 μm



(b)

500 μm

Hatching occurs through the operculum (cap).

A cluster of aeropyles (pores) allow gaseous exchange.

The egg is attached to its fibrous substrate distal to the operculum.

**Figure 1.1** Scanning electron micrographs of *Pediculus humanus*: (a) adult (b) egg



The anatomy, biology, life-cycle, physiology and epidemiology of *P. humanus* sub-species are described in [45] and references therein. Briefly, *P. humanus* are small, 2-4 mm long cylindrical insects [205] with compact, highly sclerotised thoraxes, and elongate, membranous abdomens. The cuticle may be pigmented to varying degrees [45], with recently-fed lice having a red appearance due to the ingested blood-meal. The mouthparts are adapted solely for blood-sucking [187] and consist of concentric piercing and sucking stylets with a central, long hypopharyngeal tube which delivers saliva. The mouthparts lock on to the host skin very tightly during feeding, owing to a series of tiny teeth-like structures that function as hooks [148]. The blood is sucked into the oesophagus by cavitation of the muscles constituting several pumps situated in the louse's head; this builds up the high negative pressure required to draw the viscous substrate up through the narrow feeding canals. As the energy required for suction is high, lice with free access to host skin tend to take small, frequent feeds, 5-6 times a day [45]. Louse saliva, injected during feeding, contains anticoagulant to prevent blood clotting in the feeding tubes, digestive enzymes and also a red-cell clumping agent [160].

The eggs of *P. h. capitis* are creamy-white and so blend in against the scalp [160]; they feature a cap-like structure, the operculum, which bears complex pore structures termed aeropyles. When lice hatch out, the empty eggshells they leave behind, or “nits” [45], are highly refractile, whereas embryonated eggs are not. An ignorant host is therefore distracted into focussing on nit-removal, leaving the eggs intact [159]. Although the term ‘nit’ is often used to refer to the egg, or the louse itself, authors now prefer to reserve the term specifically for the eggshells. Louse hatchlings, or ‘nymphs,’ are pale and partially transparent, but attain a fixed colour at the time of the first feed, the degree of colour varying from light to dark grey, depending on the host hair colour [160].

### **1.3 The life cycle and population structure of *Pediculus* species**

All lice undergo incomplete metamorphosis, i.e. the form emerging from the egg is extremely similar to the adult [187]; there is no pupal stage. Both *P. humanus* sub-species have three nymphal instars (stages of development), each lasting 3-5 days, before the third moult, which unveils the adult louse. Females mate within 2 days of reaching adulthood [45] and begin laying around 6 eggs per day [205] soon after. The eggs are chemically

bonded to single hair shafts, close to the roots (or fabric fibres in the case of clothing lice), by a clear, quick-setting, glue-like substance produced in the females' accessory glands [45,81]. Hatching normally occurs in 6-9 days [45] and is achieved by the nymph taking in air through the aeropyles and expelling it behind. The resulting pressure forces open the operculum and ejects the louse from the shell, the nit remains fixed to the hair. The strength of the glue by which the nit is attached results in it remaining on the hair for months or even years [160].

Many aspects of louse biology and behaviour are dependent on the stable environmental conditions provided by the host. Hatching rates can be as high as 90 % at 34 °C but fall to less than 10 % at 22 °C [205]; instar duration is also affected by variable conditions. Adult lice prefer to reside within a certain climatic range; therefore in warmer countries or more humid situations such as during host exercise, lice may wander farther afield than their usual half inch from the scalp [246].

Once a person has contracted lice, their numbers will increase if unchecked [81], however, most cases of pediculosis capitis (the condition of head lice) feature an infestation with only 5-15 lice [205], as individuals are constantly being lost by death or transmission. Lice kept *in vitro* can survive as long as 30 days, but in the wild lifespan is shorter and highly variable. Death usually occurs by physical or chemical destruction or as a result of host immune reaction rather than from old age.

The small number of lice involved in the vast majority of Western cases of head lice today is the reason why infections are so difficult to detect [59,123]. However, extremely high levels of infestation are not unknown, especially historically: In 1917, Nuttall noted a case in an infirmary where a patient had over 1,000 lice on her scalp, and workers at the institution informed him that the infestation was quite mild in comparison with those of other admittees [187].

#### **1.4 Symptoms and consequences of *pediculosis capitis***

The majority of cases of pediculosis capitis are symptom less [164] and, for this reason, diagnosis is usually made solely upon discovery of live lice on the scalp (not by the presence of eggs, as these may be non-viable or nits mistaken for eggs) [81]. In Western societies, social factors provide the main impetus for head louse removal on an individual basis. Entomologists report an 'entomophobic reaction' of shock and revulsion when lice

are discovered [81]. This is perhaps understandable for someone living in the developed world at the present time, where a high degree of control over both cosmetic appearance and contact with arthropods is expected. They may also fear that others will notice their miniature invaders, and worry that this could lead to social exclusion. The association of pediculosis with uncleanliness is common, but studies suggest lice are just as likely to infest clean or dirty hair [67,81], and overly dirty or greasy hair tends to inhibit colonisation [160]. Maunder linked the popularity of shampoo formulations with the perception of de-lousing as a cleansing procedure. However, he also asserts that although washing the hair with ordinary shampoos does not control lice, the associated grooming that goes with a certain level of personal hygiene is very important [159].

If a child has lice, its parents are likely to be the most anxious party. A Mori poll once revealed that pediculosis capitis was the childhood disease about which parents were most concerned [164]. In the USA, the 'no nit policy,' where children are banned from attending school until all lice and 'eggs' (generally not discriminating between eggs and nits) have been removed, is becoming more frequently implemented [266]. Yet it is not only children that harbour and transmit lice, as commonly believed. In fact 30 - 40 % of pediculosis capitis cases occur on adults [164]. Exclusion of children from school is likely to only cause further distress within the family, especially as parents find egg or nit removal difficult [45], aside from the fact that removal of empty cases is a pointless task.

The symptom traditionally diagnostic for the presence of lice is itching. However, studies suggest that itching, due to irritation (pruritis) of the scalp, only occurs in 14 - 36 % of cases [73,179]. Allergic reactions to louse saliva, rather than the ambulatory activity of the louse, are the cause [59]. The symptoms pass through the classical range of immune responses: no reaction, delayed reaction, immediate reaction, tolerance [179]. Because pruritis may not occur or, if it does, may take up to 3 months to develop [212] many people who have lice are unaware they have them. Maunder estimates that 2, 000 to 100, 000 louse bites are required for primary sensitisation [160] so, with an average of 10 lice present and assuming each louse feeds five times a day, it would take between 40 and 2,000 days for the sort of infestation seen in Western societies to cause sensitisation. When pruritis does occur, the irritation can become quite intense [81]; Burgess reports having seen patients who have removed considerable areas of skin by constant scratching [45].

In general, the vast majority of cases of pediculosis capitis in the West today have little or no impact on the patient's health, which causes it to be viewed as a mild complaint by the

medical profession. However, these infestations are small and short-lived; complications often occur in higher density or persistent cases.

In prolonged infections of *P. humanus*, lasting over 12 months, a systemic allergic reaction may develop, characterised by rashes distal to the site of infestation, a feverish state, heaviness of limbs, stiffness of muscles and a constant sense of malaise and lethargy, from where the term “to feel lousy” probably originates [179]. Maunder reports that, in the developing world, often the majority of children have lice almost permanently and many exhibit to some degree the apathy, enervation and low-grade anorexia symptomatic of prolonged pediculosis [163] (although his observations are merely associative in this case).

Other known consequences of louse infestation include lymphadenopathy, contracted via infected bites [2,179], allergic reactions to louse faeces, such as pseudo-elephantiasis of the ear [156], and exudative crusting of the scalp [6]. Pediculosis capitis often precipitates impetigo and pyoderma (bacterial skin infection) [45]. These conditions develop when excoriated bite lesions are colonised by bacteria or fungi from either the normal skin flora, contaminated fingernails, the bodies of lice or louse faeces [81]. Kidney inflammation (glomerulonephritis) can result if the bacterium is a nephritogenic strain of *Streptococcus* [179].

Lice are the sole vectors of classical typhus (an acute infectious fever). The infective organism, *Rickettsia prowazekii*, is spread by ingestion with the blood meal and expulsion in faeces [59]. Head lice are epidemiologically insignificant vectors of this disease in comparison with clothing lice because their faeces do not build up in large enough deposits next to the skin [159]. Louse-borne relapsing fever is transmitted when louse-gut rupture causes insect blood, infected with the spirochete *Borrelia recurrentis*, to enter excoriations in the host skin. Head and clothing lice are equally good vectors of this condition [59]. Fortunately, these diseases are both treatable with antibiotics, and are currently restricted in geographical range [45]. However, the results of past epidemics of typhus have left Maunder to speculate that human lice may have killed more people than any other insect, perhaps with the exception of malaria-carrying mosquitoes [160]. The question of whether lice can transmit HIV has been raised, but it appears that arthropods in general make unsuitable vectors for such viruses [284].

## 1.5 Transmission and epidemiology of *Pediculus humanus capitis*

Live *P. b. capitis* normally only occur on head hair although they have, on occasion, been found on eyebrows [81]. It is generally accepted that adult lice spread primarily by walking from one head to another. Lice cannot jump, although they can be thrown through the air by static charge during grooming [45]. Infant lice don't move very far on the scalp [52], so neither they nor the firmly cemented eggs would serve as dispersive agents. Only older juveniles and young adults are mobile.

Maunder believes that host-dependency prevents a louse from willingly leaving a scalp unless there was another in the immediate vicinity [159], although he has also asserted, in a somewhat contradictory fashion, that lice experience a 'powerful urge' to colonise new habitats [160]. Other sources claim that disturbances of the hair stimulate active dispersal [48]. Lice possess temperature-sensing mechanisms in the antennae that enable them to actively remain within the 31°C zone surrounding the host, which equates to a few mm from the scalp, where they live and lay eggs [164,205]. It is therefore easy to see the logic behind the claims that lice are generally passed from one person to another by relatively prolonged (30 s approx.), head to head contact [49], and that transmission usually occurs between people who know each other well [81].

Transmission of lice is undoubtedly by personal physical contact, but whether or not fomites (objects capable of carrying infective agents from person to person, for example, bedding, towels, combs) transmit lice is contentious, and has not been investigated experimentally. Maunder believes that lice found on objects other than human heads are non-viable [205]. This is probably true given the ferocity with which they cling to fibres when disturbed, their fragility, and their relative immobility on media other than human hair (on suitable media, adult *Pediculus* can travel 1 metre in less than 3 min [187]). Perhaps in warmer conditions, when they stray further [245], lice may contact intermediary objects between heads, for example by walking across pillows in the night, but re-establishment would depend on the louse not becoming damaged or dehydrated in the interim.

Human pediculi occur in all parts of the world [187] and on all age groups [5]. Children are important in the epidemiology of *P.b. capitis* because they spread lice between families or social groups through contact at school [122]. However, the reason control programmes centred on school-children usually fail is that, once in a close social group, lice can spread to all the members, and deloused children simply get reinfested. The highest prevalence of

*P. b. capitis* occurs in children aged between 4 and 11 [81] and in all age groups head lice are more common on females [168]. Social behaviour is thought to be an important determinant of head louse epidemiology, as females are more liable to engage in close contact with others, and children are more tactile than adults. Adult men also have a much greater number of empty or resting hair follicles, and head lice do not colonise widely spaced hair efficiently [160]. Hair length does not appear to affect the chances of catching lice (as put forward by Nuttall [187]) nor the numbers in an infestation [45,205]. Only when hair is shorter than a few millimetres will colonisation be affected; hair-shaft diameter, degree of curl and follicle spacing are probably more important [160]. Although Rasmussen claims that middle-class Caucasians are the most commonly affected group, lice being rare on afro-Americans [205], his evidence was probably taken from what was or is a predominantly Caucasian population, as sub-populations of lice are adapted to colonising different types of hair [45].

It appears that any situation that increases the frequency of head to head contacts will increase the frequency of infestation, and reinfestation [187]. Low socio-economic indicators such as large family size, bed sharing, crowding and inattention to medical care have been identified by some authors as factors promoting louse transmission (in fact the results of correlative studies have been very variable [45]); poor hygiene is probably not a factor, for reasons discussed previously. In this country, head lice used to be most prevalent in working-class areas of cities. Gradually the balance tipped so that, after 1976, incidence was highest among rural, suburban and middle-class children. It is believed that the gradual rise in standards of living in this country saw a decrease in the 'poverty' factors described above in poorer households whilst, in better-off households, the lessening of social formality keeping adults at a distance and increased travel to see a wider circle of friends encouraged transmission [160].

Quoting statistics to illustrate the changes in head louse prevalence over the last Century presents a problem. Early surveys, mostly derived from hospital or clinic records, lacked specific inclusion criteria of what constituted an infestation [45]. A few scientific studies were made. The incidence of head lice in developed countries in 1941 was estimated at 30 – 50 % of the human population [168]; and this figure may still be applicable to countries where commercial pediculicides are not available [45]. Years of insecticide use in this country was presumably the main reason for a much lower incidence here in 1986: British Government statistics suggested only 1.29 % of the maintained school population in England and Wales had contracted head lice that year, although these were criticised as

being over-optimistic by one author [122]. In 1993, Maunder observed that infection rates had fallen substantially in recent years [164], however, it was shortly after this that pediculicide treatment failure became a big issue. An unpublished survey of head lice prevalence around rural and urban areas of Cambridgeshire suggested that the average incidence was 7.4 % for the late 1990s [51].

## **1.6 Methods to control *Pediculus humanus capitis***

### **1.6.1 Historical aspects of louse control**

Before the introduction of chemical control in the last century, treatment of pediculosis often just involved physical removal of lice and eggs by combing, picking, or shaving [45]. Because lice were associated with dirtiness, soap and water or plain shampoo were often employed. Yet detergents only dislodge dead or dying insects and healthy lice can survive under water for up to a day. General grooming may help to keep the numbers down, but the teeth of most combs are too widely spaced to be completely effective and are not applied to the roots where lice and eggs reside [205].

Sometimes crude chemical-based remedies were employed, based on either botanicals, inorganic poisons or petroleum based organics. Mineral oils such as kerosene have long been used to kill lice and have been shown to obstruct the insect tracheal system, but in practise they have low efficacy and can also be highly flammable [45]. These treatments are still used extensively in the developing world because of the cost of Western medicines [81].

In the 20<sup>th</sup> Century, scientific research and industrial development led to the discovery and manufacture of more effective, largely synthetic, insect-specific pest control agents. The safest of these were employed in pharmaceutical preparations for the control of personal parasites. Speed of action is an important facet of insecticidal efficacy and, given that interruption of neurotransmission is one of the fastest ways to metabolically disable an insect, it is no surprise that all of the most widely used insecticides act on targets in the insect nervous system. Most neuro-active insecticides fall into one of five chemical classes [163] and generally these structural differences reflect distinctions in mode of action. The classes are therefore referred to as ‘resistance groups’ because if resistance develops to one insecticide in the group it usually extends to all the rest, although cross-resistance between

the groups also occurs. The following sections briefly describe the resistance groups, focusing on those insecticides which have been most important in louse control.

#### **1.6.2 Resistance group I pediculicides: DDT and related compounds**

DDT (dichlorodiphenyltrichloroethane) was hailed as the greatest advance yet for the control of disease vectors upon its introduction towards the end of the Second World War. However, compared to modern pediculicides it is slow acting with poor ovicidal activity. It has no residual action except on fabric. The detrimental effects of DDT on wildlife and the environment and the spread of DDT-resistance in lice resulted in its withdrawal as a pediculicide in North America and Europe, but low cost has maintained its use in poorer countries. It is less hazardous to man than popularly believed [45,81,141,163]. The neuronal target of DDT (and pyrethroids, see Section 1.6.6 for details) is the insect voltage-sensitive  $\text{Na}^+$  channel.

#### **1.6.3 Resistance group II pediculicides: The cyclodienes and cyclohexanes**

Some cyclodiene insecticides are environmental hazards, but others, such as dieldrin, are safe for public health use. Only lindane ( $\gamma$ -hexachlorocyclohexane,  $\gamma$ -HCH) remains important for louse control [163], however it is no longer used for the treatment of pediculosis capitis in the U.K. [81]. Experiments and widespread generalised use indicated the efficacy and potency of this organochlorine as a pediculicide [205]. The first laboratory studies found it to be considerably more toxic to lice than DDT [56]. However, it needs careful formulation for satisfactory ovicidal activity [163]. In comparison to the organophosphate malathion it has poor ovicidal activity and is relatively slow to act. In one experiment, 1 % lindane, the concentration of use, took 190 min to kill all lice, various pyrethrin-containing products took 10-30 min, and a malathion-containing lotion took 4 min [167].

Lindane was introduced as a general insecticide in the late 1940s [45] and, following reports of DDT-resistant head and body lice, soon became the standard treatment for pediculosis [205]. In 1983, lindane shampoo was documented as the most widely used pediculicide in the U.S. [141]. Concerns that lindane was losing efficacy arose in the 1980s, and widespread low-level resistance was found before its withdrawal from use against pediculosis capitis



[45,205]. Nowadays, lindane is available for other forms of pediculosis apart from capitis, with the trade names “Lorexane” and “Quellada”.

The primary mode of action of cyclodienes and cyclohexanes is the perturbation of inhibitory neurotransmission via the GABA ( $\gamma$ -aminobutyric acid) receptor-Cl<sup>-</sup> channel complex [183]. These compounds are not selective for the insect GABA receptor and have similar effects at the human equivalent. However, although lindane passes relatively readily through human skin, it accumulates, usually harmlessly, in body fat where it has a half-life of 3 months; nevertheless there are contraindications against use by certain patient groups [161]. Concerning adverse effects via transdermal absorption from pediculicidal shampoo formulations, the peak mean blood level following a single application to the scalp was measured at 1/30 those following use as a scabies treatment [205]. Several prominent physicians questioned the safety of lindane, but these concerns were largely unfounded given the relatively few reports of neurologic toxicity [205], which have occurred following accidental ingestion or overuse [245].

#### **1.6.4 Resistance group III pediculicides: The organophosphates**

The organophosphates have suffered from bad press in recent years, especially with respect to their use on children for louse control. Although some organophosphates are hazardous, others are among the safest insecticides known. For example, Temephos is used for the control of mosquito larvae in drinking water [163].

Malathion was introduced in 1971 and out-performed lindane in eliminating DDT-resistant lice in the field [22], it was also effective against lindane-resistant lice [205]. Various workers found it to be the first pediculicide with significant ovicidal activity, furthermore, if malathion is left on the hair for more than 12 h before washing, a residual activity persists. It was once believed that these two novel attributes would eliminate the usual need for further applications or nit combing after treatment. However, the majority of field studies suggest that, even in various formulations, the ovicidal activity is not 100 % complete, and a second treatment is recommended to kill nymphs hatching after the residual action has worn off [43]. The high pediculicidal and ovicidal activities demonstrated with 0.5 % malathion lotion [205] can be achieved with application times as short as 4 min. Malathion is available in formulation as “Derbac-M,” “Prioderm” and “Suleo-M.” One *in vitro* test identified Suleo-M as the most effective malathion-containing lotion for head lice as it had

by far the best ovicidal activity (the test was not adequate to distinguish them on the basis of pediculicidal activity) [43]. The first report of resistance to malathion was in body lice in 1969 [205]. Since then there have been a few reports of isolated cases of malathion-resistant head lice and one cluster in southern England [81].

Most organophosphates, including malathion, inhibit acetylcholinesterase (AChE). In insects malathion is oxidised to malaoxon, a more potent anticholinesterase [60]. AChE inhibitors result in over stimulation of nerves due to raised levels of acetylcholine in insect synapses. In insects, this only occurs in the CNS (central nervous system), as their peripheral synapses use L-glutamate instead of ACh [160]. Malathion penetrates human skin far less readily than it does insect cuticle [161] and, in humans, malathion is rapidly hydrolysed to excretable products or subject to attack by phosphatases which break it down into harmless products. Only resistant insects are capable of rapid hydrolysis of malathion [161]. Pharmaceutical grades of malathion are safe for use as pediculicides; they are widely used in head louse control and have a high record of safety [163] based on correct usage. Repeated or excessive application, or the misuse of agricultural grade malathion, impure forms or inappropriate formulations can result in poisoning [45,205,161].

#### **1.6.5 Resistance group IV pediculicides: The carbamates**

Again, some carbamates, including the botanical agent physostigmine, are hazardous chemicals. Carbaryl and propoxur are the two most used in head louse control, and are safe for this purpose [163]. Carbaryl lotions were introduced in 1976 [42] but use was initially largely confined to Europe [163]. Carbaryl is fast acting [163] and field studies proved its efficacy against lice, even malathion-resistant ones [205], but also showed it to be incompletely ovicidal [45]. Carbaryl products have suffered criticism for inadequate efficacy, but Burgess claims this is unsubstantiated and probably precipitated by a lack of residual effect [722], which means patients can be reinfected by undiagnosed carriers, and poor product usage [42]. Although resistance to carbaryl is theoretically possible, as shown by the ease with which a resistant strain could be selected for in laboratory lice [68], carbamate resistance has not yet emerged in field strains [81].

Formulations of carbaryl appear to be more efficacious than the isolated active on adult lice but less so on eggs, as other components of the formulations reduce carbaryl penetration,

especially over short time periods. Carbaryl is available in various formulations with the trade names Carylderm, Clinicide, Derbac-C, Suleo-C. In 1990, Suleo-C was shown to be the most effective ovicidal formulation and was one of two best at killing adult lice [42].

Carbaryl is a reversible acetylcholinesterase inhibitor, attacking the enzyme differently from malathion [48,161]. It does not readily penetrate human skin and any doing so is quickly degraded to 1-naphthol. Carbaryl has been suspected of being carcinogenic in humans at high doses, and, for this reason, the Department of Health made it available only on prescription.

#### **1.6.6 Resistance group V pediculicides: The pyrethrins and pyrethroids**

Pyrethrins and pyrethroids are particularly important in the context of this study, as they are the result of a successfully exploited natural resource; historical usage was followed by commercial production of refined and standardised plant material and, later, semi-synthetics based on the active constituents were developed. Their development and use in personal parasite control is summarised here. The rethrins are insecticidal esters occurring in *Chrysanthemum cinerariaefolium* Vis., and *C. coccineum* Willd. [135]. They are formed from the combination of chrysanthemic acid or pyrethric acid with the three alcohols, pyrethrolone, cinerolone and jasmolone to form two sets of three rethrins, pyrethrins I and II respectively. In Persia (now Iran), the insecticidal properties of plants of the *Chrysanthemum* genus were known for centuries, but were kept secret. Then, in 1840, commercial production of *C. cinerariaefolium* flower heads began in Dalmatia (now Yugoslavia). The use of the powdered flower heads spread quickly through Europe and, in 1860, reached the USA. At the end of World War I, three million lb per annum were being used [135,246].

Between 1919 and 1929, it was first noticed that kerosene extracts were more active than the dried flowers. The active components were named 'pyrethrin I and II,' and extracts were then standardised on the basis of these markers. By observing the relative levels of these marker compounds according to environmental conditions, one USA company identified which parts of the world would grow the most insecticidal pyrethrum plants [246]. Success of rethrin-containing products as insecticides was curtailed owing to inadequate supplies of pyrethrum, the introduction of DDT, low photo-stability, and rapid metabolism in insects, which limited their potency and application. Pyrethrum extract was also expensive compared to synthetic insecticides, although patenting of the synergist

piperonyl butoxide in 1949 meant that manufacturers could use less in formulations [246].

Crude pyrethrum flower extracts are refined, and standardised such that commercial products contain 0.17 - 0.33 % pyrethrins, the rethrin composition being variable depending on the type of flower and the extraction process used [205]. Such products have been criticised for their variable quality, poor ovicidal activity, and chemical instability [163]. Professionals tended to regard pyrethrins as having low efficacy, but poor formulation was probably to blame in many cases [45]. Pyrethrins, formulated with a synergist in a shampoo vehicle, were tested against head lice and performed well in comparison to malathion but were not as efficacious as permethrin [45]. A study by Meinking and Taplin showed that one fresh pyrethrin combination had very rapid killing time (10 min) but a 2 month old product took 30 min to achieve the same result [167]. All pyrethrin formulations so far available have had particularly low ovicidal activities [45,163,205].

Both pyrethrins and piperonyl butoxide are poorly absorbed by human skin. Side-effects from pyrethrin use are extremely uncommon and are usually some form of skin reaction due to other formulation or extract components. Pyrethrins do irritate mucous membranes, and contact with eyes should therefore be avoided [205,163]. More stable, effective and selective chemical relatives of pyrethrin have been investigated since the development of the first synthetic pyrethroid in 1949 and, in 1987, Taplin and Meinking reported that over 50 pyrethroids had come into use for pest control [246]. Pyrethroids include (S) – bioallethrin, tetramethrin, resmethrin, phenothrin, kadethrin, permethrin (the first photo-stable pyrethroid) and decamethrin [135]. Again, these range in toxicity, the safest being selected for louse control. In 1991, Maunder reported that phenothrin was the most popular pyrethroid in Europe; permethrin was preferred in America [163]. After the development of pyrethroids the pyrethrins were viewed as inferior, they have remained in use because they are currently relatively cheap in comparison to synthetic insecticides. Pyrethroids can be made far more economical when formulated with synergists, but the British Committee for Safety of Medicines are wary about the use of synergized insecticides on humans [163].

When permethrin was first used in a product (NIX® 1 % permethrin crème rinse), in the USA in 1986, it was found to produce complete cure, even though ovicidal activity was incomplete [246]. This was due to the residue of permethrin which persisted on the hair long after the conditioning vehicle had been washed off [246].

The toxicity margin for permethrin is good and post-marketing surveillance studies showed it to be relatively side-effect free [10], it is photo-stable, and actually less toxic to animals than pyrethrins, hence its choice for personal parasite control [246]. Pyrethrins and related compounds are known to undergo rapid metabolic degradation in mammals and birds [135], probably via esterase activity in skin [246]. Burgess believes that the low concentrations of pyrethrins, permethrin and phenothrin in commercial formulations also partially contribute to product safety [45]. Side effects may occur if the patient has a predisposing allergy to *Chrysanthemum* plants, or other members of the *Compositae* family, such as ragweed, but it is thought that the major allergens are removed by modern refining techniques [246]. Skin sensitisation [161] and cases of acute irritancy have mostly been attributed to excipients [45].

Many insect species developed resistance to pyrethrins and pyrethroids fairly quickly after their introduction [161]. Lice are no exception, and permethrin-resistant pediculi are already widespread in Britain [47], first occurring within 3 years of introduction [48]. Permethrin resistance has been documented in Israel and the Czech republic, whilst anecdotal reports have come from a number of other countries [46]. One study has shown phenothrin resistance in France [66]. It is expected that this resistance will render useless any product containing permethrin, phenothrin, pyrethrins, or related compounds. However, although pyrethrin resistance was demonstrated in 1955 in body lice (no cross-resistance with lindane or malathion is believed to occur), so far there has been no documentation of pyrethrin-resistant head lice [205].

Pyrethroids and pyrethrins have the same neuronal target as DDT and both types of compounds produce a rapid paralytic (knockdown) activity. Pyrethroids first stimulate nerve cells and fibres to discharge repetitively; this then leads to paralysis from exhaustion of neurotransmitter. The repetitive discharge is mediated by them binding to the voltage-gated sodium channel and increasing the duration for which it is open [183]. This prolongs the sodium current, which in turn elevates and prolongs the normal depolarising after-potential. This causes the membrane to reach the threshold for action potential again, thus initiating repetitive after-discharges. Pyrethrins and pyrethroids are selective towards insects mainly because invertebrate sodium channels are more sensitive to them than are the vertebrate equivalents, and due to the negative temperature dependence of pyrethroid action on the sodium channels (the flux increases at low temperature); pyrethroid degradation has only a minor role [183].

### 1.6.7 Miscellaneous louse control methods

Treatment failures after the use of approved synthetic and semi-synthetic chemical pediculicides, and the current antipathy of certain sectors of the general public towards them, have led to research into new avenues of lice control, often turning to ‘traditional’ methods for inspiration. In the U.K., “BugBusting” is a regime for physical removal of lice invented by the charity Community Hygiene Concern and involves regular, thorough wet-combing of conditioner-covered hair. The comb should be plastic and have teeth not more than 0.3 mm apart [52]. Such ‘nit combs’ or ‘detection combs’ (for they are also used in diagnosis) can remove even the smallest first instar nymphs and may also remove eggs or nits [48]. Burgess suggests that this alone is unlikely to be efficacious as it can take up to 9h of thorough searching and grooming to be sure that all lice, eggs and nits are removed and few people have sufficient motivation or skill to produce a complete cure [45].

Some chemicals have been investigated for their nit/egg-removing potential. Vinegar was used in lice treatment for many decades and was supposed to detach louse eggs, however, studies have proven it to be ineffective for this purpose [59]. Formic acid was proposed on the strength of its reputed chitin-dissolving properties. Unfortunately, recent studies suggest that the substance attaching lice eggs to hair is non-chitinous [53]. Preliminary *in vitro* work with the ‘slip-peel test’, used by the MEC to identify effective nit-removing agents, which decrease the amount of force required to dislodge a nit from a strand of hair, also indicated its inefficacy [45].

Substrate removal was the biggest factor contributing to the near extinction of clothing lice in the developed world: People who change their outfit daily do not harbour clothing lice, as the lice cling to the removed garments and cannot tolerate the ensuing periods of starvation, low temperature and low humidity when the clothes are not being worn. Unfortunately, the equivalent for head lice would be to cleanly shave the entire head and neck area of infected families, therefore this method of head louse control is unlikely to become popular.

In his 1995 review [45], Burgess highlights some potentially new avenues in pediculicide research: For example, some systemic drugs, taken for other purposes, appear to incidentally affect the survival of head lice. These include selected non-steroidal anti-inflammatory drugs, certain antibiotics and ivermectin (avermectin B<sub>1</sub>). New possibilities for topical application include natural and synthetic insect growth regulating compounds,

and the imidazoline miranols, short-chain surfactants which were found to specifically remove lipids from the water-proofing layer of louse cuticles.

## **1.7 Shortcomings of head lice treatments**

At the present time, those with pediculosis capitis are finding it very difficult to treat successfully. There are numerous shortcomings associated with head louse control methods and they, along with the resilience of lice and their eggs, make eradication prone to failure.

### **1.7.1 Insecticide resistance**

Insecticide resistance occurs due to artificial selection (insecticide use is the sole selection pressure driving its development): when a population of lice is subjected to a certain dose of a particular insecticide which would normally be lethal, and at least one louse survives, owing to a difference in genetic make-up, this louse will survive to pass on its 'resistant' gene, or genes, to its progeny [74,163]. The 'resistant' forms of the gene(s) will therefore be propagated among populations of lice in favour of the normal or 'wild-type' gene(s), owing to the preferential reproduction of individuals with the resistance phenotype over sensitive individuals. The extent of genetic interchange between insect populations determines the total proportion of the species that will acquire the resistance allele(s) [74] and the reproduction rate determines the rapidity with which the resistance will spread.

Resistance has either occurred naturally or been demonstrated possible for each resistance group listed above. This is not surprising given the history of insect adaptability towards these chemicals. As recently as 1986, Rasmussen believed that resistance in head lice was uncommon and that poor ovicidal activity was the cause of most pediculicide treatment failures [205]. Since then, resistance has increased to the point where its impact on head louse control is indisputable [149].

Resistance can occur due to modification of factors determining uptake, target site interaction and metabolism. Resistance to carbamates and organophosphates has often been shown to correlate with the insensitivity of AChE variants to these insecticides in various species, for example, in a documented incidence of methomyl (a carbamate) toxicity towards wild strains of the beet armyworm, *Spodoptera exigua*, no cross-resistance

with chlorpyrifos (an organophosphate) was found, emphasising the subtle difference in mode of action of the two types of AChE inhibitor [60]. By contrast, a variant AChE, encoded by the allele *AceR* in wild *Culex pipiens quinquefasciatus* was found to confer cross-resistance to both organophosphates and carbamates [63].

An interesting breakthrough has been made regarding resistance at the DDT/pyrethroid target. cDNA clones containing part of a housefly Na<sup>+</sup>-channel gene, *Msc*, were found to generate different lengths of DNA when subjected to digestion by the same restriction endonuclease enzyme. This technique is used to identify sequence variation between closely related sections of DNA, usually different alleles of the same gene, or evolutionarily related genes. Related sequences that differ with respect to the pattern of digestion are said to contain Restriction Fragment Length Polymorphisms (RFLPs). Inheritance of *Msc* RFLPs showed tight linkage to inheritance of a form of pyrethroid (and DDT) resistance, 'knock-down resistance' (*kdr*) [267]. This study provided perhaps the most firm evidence so far that the pyrethroid target mediating toxicity is the voltage-gated Na<sup>+</sup>-channel. Confirmation of the molecular target of dieldrin and related compounds was also achieved by the observation that variations in resistance phenotype correlated with certain changes in the genome. The Maryland strain of *Drosophila*, which is dieldrin-resistant, was found to contain a mutation in a GABA receptor subunit gene, *Rdl*; cloning and expression of both wild-type and mutant RDL subunits later confirmed that the GABA receptors they formed *in vitro* were differentially susceptible to dieldrin [87,88].

Some resistant insect tissues contain elevated levels of microsomal oxidases; these enzymes detoxify insecticides, thereby lowering the dose reaching target internal organs. Piperonyl butoxide inhibits microsomal oxidases and is often used in formulations to increase the insecticidal potency. Other enzymes can also contribute to resistance, including hydrolases, esterases, glutathione transferases and dechlorinases. Enzymatic mechanisms such as these can give rise to cross-resistance phenomena (as has been seen between dieldrin and lindane or between organophosphates and carbamates), as they are often non-specific in action, and can inactivate multiple chemical structures [74].

In head lice, cross-resistance via enzymatic detoxification mechanisms is thought to underlie the reason why both organophosphate and pyrethroid resistance currently exist without decreased efficacy of carbaryl [48]; both malathion and pyrethroids are normally metabolised by esterase enzymes in all insects, whereas carbaryl is metabolised by oxidases.



As insect populations have proven highly responsive to selection pressures from insecticide use in the past, it is important to ensure that any new compound is used wisely to remain effective. It has been suggested that the concomitant [74], rotational or mosaic [45] use of at least two insecticides (presumably with different targets) is preferable, as the probability of two different resistance types existing in a single population is much less than for one [74]; the drawback to this strategy is that alleles conferring cross-resistance render it less viable. Also, as with antibiotics, prophylactic use is not recommended, as this increases the selection duration [163].

Unfortunately, the resistance-avoidance application regimes mentioned previously are impossible to implement, as most pediculicides are OTC medicines. Up until the start of the 1990s, the majority of U.K. health authorities rotated recommended pediculicides on an annual or triennial basis, this being possible because clinics had a monopoly on their supply. When this was dropped in 1992, rotation became unenforceable [51]. Since then, mosaic prescribing (the simultaneous availability of insecticides from several different resistance groups) offers the best means of preventing resistance development [45].

It could be argued that new insecticides are not needed, but instead, a more judicious use of what is already available. However, in reality the ability of such application strategies to keep resistance at bay is contentious, due to the existence of both monogenic and polygenic resistance genotypes, the latter developing over a long period of time in response to multiple different insecticides. For example, resistance to organochlorines usually involves a single gene and there is little cross-resistance between organochlorines and other compounds, even between the closely related DDT and lindane. In contrast, resistance to organophosphates spans to related and unrelated compounds [141]. Insecticide resistance is a complex phenomenon with variable components; the huge range of possible mechanisms is coupled with differing probabilities of occurrence and persistencies of resistant alleles. The widespread occurrence of DDT-resistance in lice stopped its use in many parts of the world. Some time after, it was noted that some populations of lice reverted to the sensitive phenotype, whereas others did not, presumably due to the co-existence of more than one type of resistance mechanism [141]. It should also be noted that the residual action displayed by some pediculicides, which was once believed to be advantageous as it kills hatchlings from surviving eggs and also prevents reinfection following treatment, is now believed to hasten the development of resistance [42,163] by increasing the selection pressure duration. The other disadvantage of residual action is that it eliminates lice caught by reinfection, which prevents the identification of other pediculosis sufferers by 'contact

tracing' [42].

Maunder believes the development of resistance to an insecticide is inevitable following 10-20 years of widespread use, but there still remains the possibility that some compounds exist to which insects are unable to become resistant. It could be said that a fly cannot become resistant to a swat; might it be possible to find the chemical equivalent?

Unfortunately the mechanism of action of a swat is rather non-specific, it is the application method that provides the specificity. Because pediculicides are applied to the human head, factors determining their toxicity must be as insect-specific as possible. However, the specificity of a compound can also be its downfall, as a minor change in the target site or the processes determining pharmacokinetics will render them inactive. With detailed knowledge of the mode of action, structure and metabolism of an insecticidal compound, the researcher may be more equipped to predict the likelihood of resistance, however, given the failure of a previous attempt to design a pesticide immune to resistance, such assessments may be of limited success: Insect-hormone mimics are extremely similar to the endogenous chemicals and it was therefore thought that any mutation favouring resistance would render the arthropod non-viable, yet they were subsequently overcome by insects with polygenic resistance [141].

### **1.7.2 Toxicity towards humans**

Concerns regarding pediculicide toxicity have been expressed by the general public and discussed in the popular press [81]. In fact, reports of systemic toxicity from proper use of these agents are rare [205], although isolated incidents have occurred [81]. In poor families or where Western medicines are unavailable, cheap formulations or low-grade agricultural insecticides have been used and these have resulted in fatalities [269]. As with most medicines, however, there are contra-indications, and minor side effects are not uncommon.

### **1.7.3 Incomplete ovicidal activity**

The structure of the louse egg shell is such that few molecules penetrate it. Mature eggs also effectively contain a fully formed louse surrounded by its own cuticle, and therefore have multiple protective barriers against deleterious chemicals [161]. Insecticides generally collect at shell level and kill emerging nymphs rather than the developing embryo, but this is not ovicidal action per se, and the active ingredients on their own usually fail to kill nymphs prior to hatching. Some formulations contain excipients that enhance ovicidal activity or may be ovicidal themselves [48,51]. Furthermore, very young embryos have no nervous systems and therefore neuronally active insecticides are unlikely to have any effect at this stage [45]. So far, no commercial product utilises separate pediculicidal and ovicidal agents; the usual approach is to formulate a product in which both activities of a single insecticide are optimised.

### **1.7.4 Product formulation**

The majority of pediculicidal products are lotion or shampoo formulations. Insecticidal shampoos are often criticised [41] as the short application times and the dilution factor when mixed with water (0 to 150 fold) result in doses inadequate to kill even adult lice [50]. Shampoos, however, remain popular with the public, probably because of their association with cleanliness [41,50] and the louse's association with dirtiness.

Evaporating lotions perform better because they are applied for a longer duration and also result in product being applied, undiluted, directly to the surfaces of lice and eggs [162,163]. Crème rinses, introduced more recently, supposedly combine convenience, efficacy and protection from reinfestation by residual action [45]. Their conditioning qualities leave the hair more manageable so that combing and nit removal after treatment are facilitated [246]. A mousse formulation for application to dry hair was developed by Burgess [44]; he claims it will allow a short application time, and yet also produce a high ovicidal activity.

### **1.7.5 Treatment utilisation**

Finally, it seems that louse control lacks proper utilisation of the best strategies for treatment. The public and health-care professionals alike are often not adequately educated about lice and the importance of correct pediculicide use [41], else they choose to ignore the information they are supplied; misapplication, such as too little product being applied is an important cause of treatment failure [45].

Control of lice should ideally be on a global level, not something which individual families attempt alone. The only way to effectively control an epidemic is to co-ordinate treatment within the entire social setting by involving schools and local authorities [205]. As most infections have existed for some weeks before being discovered, contacts over the last month should be traced and treated to prevent reinfection and further spread by identifying undiagnosed carriers. Unfortunately, the social stigma surrounding lice in Western societies greatly impedes effective 'contact tracing' [81], as does the residual effect of some products [162].

### **1.8 Will more pediculicidal agents help to control head lice?**

Of all the insecticidal compounds in use today in the U.K., at present there are only four recommended for use against *P. h. capitis*: malathion, permethrin, phenothrin (all OTCs), and carbaryl (POM). If the products being sold were all completely and consistently efficacious then four different active ingredients would probably be sufficient for the pediculicide market. In 1991, Maunder [163] stated it was our ability to find and prevent lice that limited control, not our ability to kill them, and that no new insecticides were necessary. However, it might seem that this situation is unlikely to hold if resistance, especially of the polygenic or irreversible varieties, continues to take hold. Cross-resistance can clearly exist between both closely related and divergent molecules, indicating that new therapeutic agents should have both a novel chemical structure and a novel mode of action to have the greatest chance of success. New insecticides should be developed not only in case resistance occurs to all the currently available actives but also to facilitate mosaic insecticide use.

## 1.9 The potential for natural products in pediculosis therapy

Remedies based on crude botanicals were used in the developed world before the invention of synthetic insecticides and are still used elsewhere for reasons of cost and availability, but in some Western societies in the last 15-20 years, there has been an increasing demand for 'natural' therapies (with plant or other natural product derived constituents), particularly among certain consumer groups [124]. People who purchase natural product-based pediculicides out of choice (rather than those who do so out of desperation following multiple treatment failures) are often suspicious of the safety of orthodox preparations and believe that remedies based on plant extracts will be side-effect free which, of course, is not necessarily true. Other consumers are perhaps not exactly sure why they prefer such treatments, and probably do so for the sole reason that 'natural' is in vogue; this is evident from the ever-increasing number of health and beauty products with names and/or packaging alluding to the contents' proximity to nature.

Scientifically proven efficacy, target-organism selectivity and medical council approval still do not appear to be primary factors in natural pediculicide use, despite the fact that such treatments have received widespread media attention in the West [48] since around the mid-1990s.

## 1.10 Plant derived insecticides

The biological activities of certain plants in insect control were known in the earliest recorded times, and some species were mentioned by writers as far back as the 5<sup>th</sup> Century BC [235]. Today over 2000 plants are known to possess some insecticidal activity [128] and in many instances the herbs have a history of use in folk medicine and are still utilised locally. This is not surprising, as the static habit of plants has necessitated their evolution of a vast metabolic flexibility to help them thrive in their fixed environment. Thousands and thousands of 'non-essential' chemicals are produced, some constantly and some in response to environmental triggers, which have proven roles in aiding the survival of plants in adverse conditions or promoting their proliferation. Many of these 'secondary metabolites' are known to affect insect behaviour and viability. Despite this huge number of potential avenues in pesticide research, for the majority of cases the use of natural products in pest control lacks large-scale commercial development [196]; notable exceptions include nicotine, pyrethrum, hellebore, and the roots of *Derris* and *Lonchocarpus*

[135].

Disappointingly, sometimes authors of scientific literature can be found making generalisations about 'natural' and 'synthetic' chemicals on the same level as the general public. For example, Palevitch and Craker state that 'synthetic insecticides have suffered a number of environmental, economic and human health problems, including persistence of residues, development of pest resistance and injury to non-target organisms' [196].

Whether intentional or not, this statement may lead the reader to assume that these problems are characteristic of synthetic insecticides, and that natural products all fall into a specific category of chemicals which do not have these short-comings. Perhaps the only generalisation that can be made is that plant-derived substances are usually highly biodegradable [135], whereas synthetic compounds may or may not be; it cannot be said that they will be less likely to induce resistance, or be more selective than synthetics. In the same way that some authors' generalisations cause them to appear biased toward natural products, those of other writers perhaps reveal a certain cynicism: the continued demand for natural rethrins despite worldwide success of the pyrethroids is put down to the trend for all things natural by Burgess [45], but Klocke argues that they are often favoured over their synthetic counterparts for use in the home because of low mammalian toxicity and lack of residues [135].

There are several ecological rationales as to why natural product insecticides might be superior to those designed by scientists. For example, it could be argued that these chemicals might be selective for particular insect species, or related species, as plants depend on non-pest arthropods and mammals for reasons such as dispersal of seeds or pollen, and their chemical defence systems must not harm them. However, specificity is not always conferred by the nature of the toxic substance, for example, sometimes compartmentalisation is used to store these chemicals or their precursors, such that only mechanical damage will elicit their release. In support of these ecological theories, the literature contains many examples of plants and their constituents that are highly specific in their lethality towards pest insects [135,196], others, such as pyrethrins, are classified as broad-spectrum.

### 1.11 Plant derived pediculicides

Head lice, of course, pose no threat to plants, yet this does not preclude the discovery of phytopediculicides. There are even ecological arguments for their existence: it has been suggested that blood-sucking parasites may be evolutionarily related to tube-feeding phytophagous insects as both groups possess similar piercing and sucking mouthparts [148]; it is therefore conceivable that chemicals lethal to sap-suckers may also be useful against distantly related blood-suckers. The other possibility is that parasites evolved from skin-eating insects, which evolved from non-keratinophagous insects living close to warm-blooded animals [148]. Such a separation from their vegetation-dwelling ancestry and a high degree of adaptation to the parasitic lifestyle means that parasitic insects may have lost any genes offering protection from plant allelochemicals, and therefore could be susceptible to these compounds.

Despite the reputed pediculicidal activity of many plants and their use in traditional medicine systems as such, there is a relative paucity of scientific analyses of these plants. In his comprehensive review, Burgess [45] describes the results of research into natural products pre-1960: A study of Traditional Chinese Medicines used to kill lice (which has provided many potential leads in phytotherapy research), found that only extracts of *Stemosa tuberosa* (pai pu) were active in tests. Busvine tested a variety of botanical materials in the 1940s and reported them to be of limited efficacy. Buxton also disproved the reputed activity of vegetable oils (the information in this paragraph is summarised from [45]).

Although the early studies of botanical materials appeared to disprove efficacy more often than demonstrate it, there were notable exceptions: In the search for a cheaper nicotine substitute in the US in the early 1900s, interest in *Quassia amara* L. arose. The wood and bark (quassia ‘chips’) of this small tropical tree had long been used against insects [135] and appeared to have a narrow spectrum of activity, being used to control aphids and sawflies as a contact or stomach poison. The major insecticidal principle is quassin, a degraded triterpenoid (decanortriterpenoid), and many related ‘quassinoids’ have been isolated from other plants of the Simaroubaceae [79]. Infusions of quassia chips are still recommended as a head louse remedy by Western herbalists, and were reintroduced on a wide scale in Denmark when DDT resistant lice emerged [130].

Rotenone was used for delousing of prisoners of war before the introduction of DDT.

Rotenone is an isoflavonoid extracted from the roots of *Derris* and *Lonchocarpus* species, which contain rotenone at 3-10 %. Rotenoids act by blocking transfer of electrons to ubiquinone by complexing with NADH dehydrogenase, thereby inhibiting respiration; they are selective to insects due to rapid metabolism in mammals when ingested (but not when entering the bloodstream) [79]. Pediculicidal studies found it slow acting and poorly ovicidal, but it did give a high mortality rate on adult lice and a cream formulation for pediculosis capitis was available in France until recently. A drawback is that rotenone tends to cause dermatitis with regular topical application [45]. Benzyl benzoate, from Peruvian balsam, persisted in mixed formulations for treatment of pediculosis capitis in some countries until recently. Early scientific evaluations gave mixed reports of efficacy [45], more recently it was shown to have reasonable ovicidal activity [32].

The most successful pediculicidal natural products so far, whether used as the active itself or whether used as a lead compound for development, are still the pyrethrins. Recently, however, there has been much discussion of the use of ivermectin as a head lice treatment both systematically and topically [178,279]. Ivermectin is a semisynthetic derivative of the avermectins, a group of macrolides produced by *Streptomyces avermectilis*. Avermectins and ivermectin display anthelmintic, insecticidal, and acaricidal properties but low toxicity to humans. They act by blocking neuromuscular transmission in sensitive organisms by acting on GABA receptors [79]. As ivermectin is already a hugely successful drug when prescribed for other purposes, it may prove equally useful in pediculosis control; however, the use of ivermectin against lice awaits approval.

Perhaps surprisingly, even the most recent entomological and phytotherapeutical literature contains very few investigations of natural products as pediculicides, despite the recent growth of interest in both lice and phytopharmaceuticals in terms of both media attention and scientific studies. Studies in the last few years have identified some pediculicidal plant extracts, for example, organic solvent extracts of *Annona squamosa* Linn. (custard apple, a Thai folk medicine ingredient) seeds and leaves have been shown to be efficacious against head lice. A preliminary cream formulation consisting of 20 % w/w of a petroleum ether extract killed 95% of head lice after a 3 h exposure to 20 g, a higher % kill than the standard drug (25% benzyl benzoate emulsion) [250]. However, aside from the most commercially successful agents, the plant derived pediculicidal substances for which efficacy has most often been reported, both historically and in modern research, are the essential oils and their constituents.



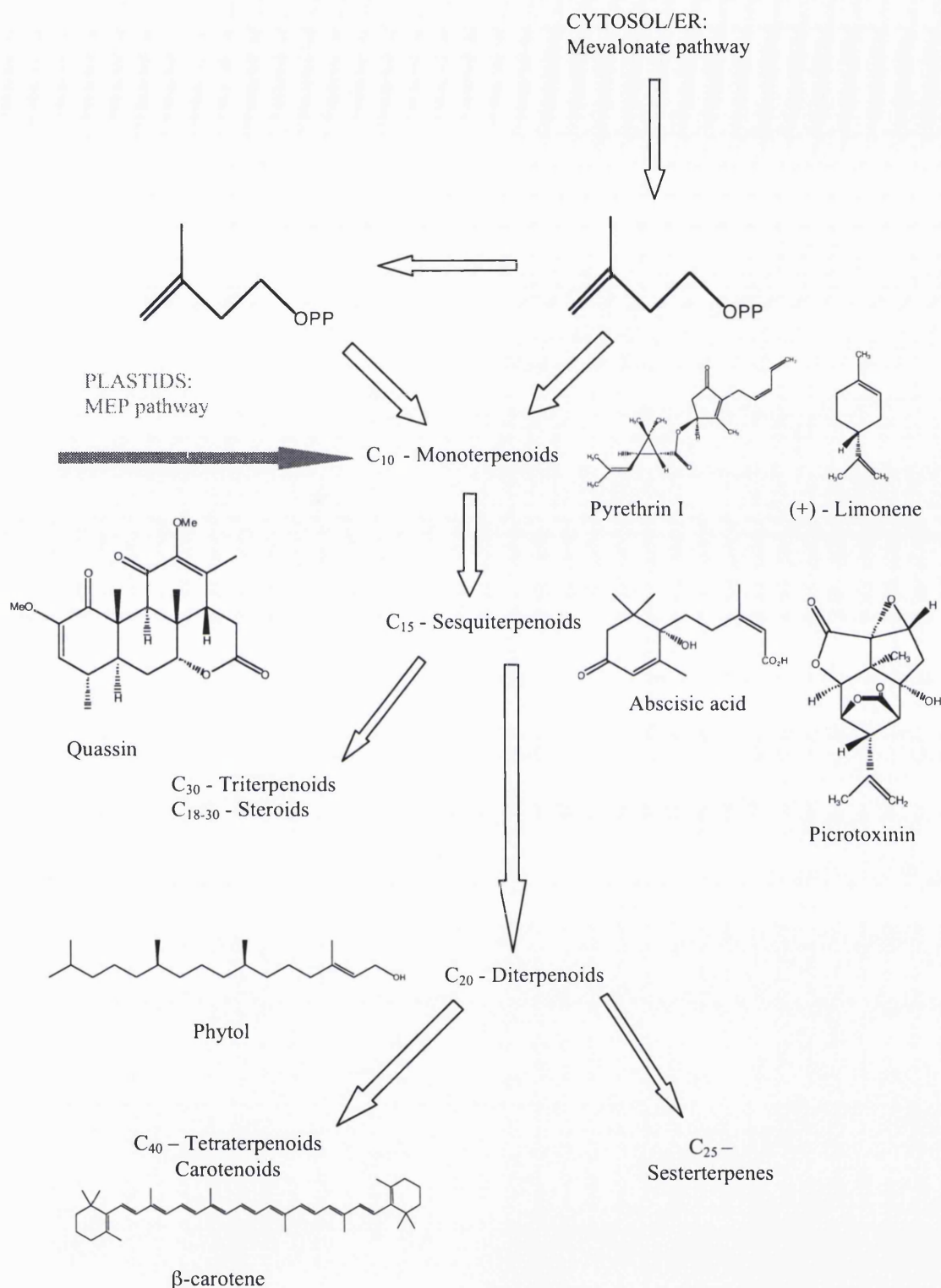
## 1.12 Essential oils and terpenoids

Plant-derived insecticidal compounds span a large number of chemical classes. For several years, particular essential oils, especially tea tree oil, have been put forward as alternative effective pediculosis control agents in scientific and media articles and are also recommended for this purpose in herbal textbooks. Essential oils and the major constituents of essential oils, the monoterpenoids, therefore provide a good starting point for investigation. Although they have been thoroughly tested on a range of insect species, scientific studies of their pediculicidal properties have not yet been very extensive. The enormous variety of essential oils and terpenoid constituents offers the opportunity for detailed analysis of structure-activity relationships and potential additive or synergistic effects.

Essential oils are plant extracts that have been used for centuries as medicines, fragrances and insect repellents. They comprise a selection of compounds, most of which are volatile at room temperature and therefore can usually be harvested by steam distillation. If any of the constituents are unstable at high temperatures then less harsh methods such as solvent extraction, or sometimes expression, can be employed [79]. The yield and chemical constitution of a particular essential oil varies according to the species and cultivar, environment and extraction methods. The composition, in turn, determines the commercial value or “quality” of the oil, and where minimum or maximum levels of certain components are required, such as in medicinally important oils, the products of extraction are selected to meet these requirements so that the oil is “standardised”. For example, Australian standard number AS 2782-1985 for Oil of *Melaleuca* (Tea Tree Oil) now requires that the terpinen-4-ol content should be greater than 30 % and the irritant cineole kept to less than 15 % to maximise the wound healing properties of terpinen-4-ol [82]. Although some essential oils, such as aniseed, nutmeg and fennel, are composed primarily of aromatic, shikimate biosynthetic pathway products, the major constituents of most essential oils are the 10-carbon-based monoterpenoids, produced via the mevalonate pathway [79]. Constituents of plant volatile oils have been known to affect the behavioural responses of pests for a long time, with the terpenoid components appearing most useful in control of numbers or predatory activity [196].

Terpenoids form a class of metabolites found in living organisms. The two known routes of synthesis and the common biosynthetic precursors define the group, to which molecules of extremely diverse size structure and function belong (See Figure 1.2). The mevalonate biosynthetic pathway was described some years ago but, more recently, a second route of terpenoids synthesis, the 2-C-methyl erythritol 4-phosphate (MEP) pathway, was discovered [153]. The MEP pathway appears to be localised in the plastids, and therefore probably only contributes to the synthesis of plastidic terpenoids, such as the carotenoids and the phytol side-chain of chlorophyll [153]. All non-plastidic terpenoids are probably derived via the cytoplasmically localised mevalonate pathway, and therefore this biosynthetic route is likely to produce the vast majority of essential oil constituents.

Plants utilise the terpenoid secondary metabolic pathway extensively. All plants produce terpenoids; there are members of the class which are ubiquitous to all plants, such as phytol; there are some which are characteristic of particular species, for example, the essential oil of tea tree (*Melaleuca alternifolia*) characteristically contains a high amount of terpinen-4-ol. Each plant species produces its own individual array of structures and these fulfil diverse roles, from the 40-carbon-based carotenoids involved in the light-harvesting stages of photosynthesis to the smaller, volatile compounds, such as mono- and sesqui-terpenoids, which give flowers their scent and aid pollination by insects [101].



**Figure 1.2 The terpenoid biosynthetic pathway in plants**

The terpenoid skeleton is based on specific C<sub>5</sub> units derived from either the mevalonate pathway, or the 2-C-methyl erythritol 4-phosphate (MEP) pathway. Some ubiquitous terpenoids have pivotal roles in plant growth and survival, such as the phytol (diterpene) side chain of chlorophyll, and the hormone absciscic acid (sesquiterpenoid); Some terpenoids are common but have secondary roles, such as  $\beta$ -carotene (carotenoid); Other terpenoids are restricted to certain groups of plants and include insecticidal agents such as (+)-limonene (monoterpenoid), picrotoxinin (sesquiterpenoid), quassin (modified triterpenoid) and pyrethrin I (formed from the monoterpenoid chrysanthemic acid).

Ecological roles have been identified for many terpenoids, and a large number appear to function in protecting the plant from pathogen attack. Many terpenoids are lethal to insects, acting in the vapour form or as contact agents [70,196]. Terpenoids and their derivatives already have a major role in the insecticide industry: The 6 rethrin esters, pyrethrin I and II, cinerins I and II and jasmolins I and II all have a monoterpene component. However, there are particular reasons why essential oils and their constituent monoterpenoids might be particularly suited to the problem of pediculosis control. At present, there is widespread use of essential oils for their scent, aromatherapeutic, repellent or other biological activities. Essential oils or isolated monoterpenoids can be found in massage oils, dental preparations, cosmetics, various pharmaceuticals and pest deterrents. Many essential oils have been used in home- and commercially-produced preparations for years. Such 'hallowed usage' tends to make obtaining a licence to market them in formulations for other purposes much easier. The basic production processes and widespread use make many essential oils and monoterpenoids cheap and easy to obtain.

The close association between essential oils and aroma mentioned above is not without good reason: Most monoterpenoids, and essential oils, are pleasantly aromatic; in fact, the unpleasant smell of the first synthetic insecticides, the thiocyanates, used extensively in the Second World War prior to DDT, was often mitigated by the addition of an essential oil [55], and they are still used for this purpose in certain formulations. Burgess states that he has found, in unpublished observations, that terpenes can be just as insecticidal and more ovicidal than conventionally accepted insecticides, and that this would explain the especially high *in vitro* ovicidal activity of Suleo-M above other malathion-containing pediculicides [43], as it also contains terpineol and *d*-limonene in the formulation as fragrance enhancers.

Essential oils generally have favourable toxicity profiles; Palevitch and Craker state that monoterpenoids exhibit low toxicity to mammals [196], but of course this is relative to the dose. Some monoterpenoids are more hazardous than others, and even the safest monoterpenoids and oils often have specific contra-indications and possible side-effects [251]. Commonly used essential oils can be obtained from health-stores in pure form, which unfortunately causes some people to view them as being innocuous and encourages misuse.

A low cost, specific, safe, acceptably scented, pediculicidal and ovicidal active component of a head lice formulation such as monoterpene(s) or essential oil(s) would be ideal to treat this common, primarily cosmetic complaint. At present, such a product would have

further appeal to consumers in the West as it could be marketed as containing “natural ingredients” or “plant extracts,” and may even be more accessible to the developed world if production costs were low.

#### ***Evidence for the efficacy of essential oils and monoterpenoids as pediculicides***

Apart from the commercially available phytochemicals and derivatives discussed previously, essential oils appear to have been of particular success amongst the pediculicidal natural products: Oil of sassafras was used historically as a pediculicide; the active, but also carcinogenic, ingredient was identified as safrol [225]. The utility of eucalyptus oil was discussed in the early part of last Century [198] and towards the end [132]. Lavender oil was used to repel clothing lice around collars and cuffs in the trenches in the First World War.

Recently, there has been renewed interest in the scientific study of essential oils and monoterpenoids as pediculicides: Neem oil from *Azadirachta indica* performed well against head lice when compared with other standard insecticidal treatments; the authors also added that although the neem oil extract was less active than malathion in terms of the  $LT_{50}$  value, they believe it to be more safe for human usage [177].

Preparations of essential oil from the leaves of *Lippia multiflora* Moldenke (Verbenaceae) were tested for knockdown activity versus body lice and head lice. Knockdown times were comparatively shorter than those obtained using benzyl benzoate and Delvap Super, a brand of dichlorvos, and the lethal effect was enhanced when applied in an enclosed system. Terpeneol was identified as a major constituent of the oil [189].

Oils of aniseed, cinnamon leaf, tea tree, red thyme, oregano, rosemary and pine were all effective against lice in a study by Veal *et al* [256], and all except rosemary and pine showed appreciable ovicidal activity. The most effective oils contained large proportions of phenols, ketones and phenolic esters, except for tea tree, which contained a high proportion of alcohols. Previous work by Gauthier *et al* identified 1,8-cineole as the active component of myrtle oil [287]. Terpinen-4-ol and  $\alpha$ -terpineol were found to be more active than  $\gamma$ -terpinene by Downs *et al* [286]; and the oxygenated monoterpene component of *Cupressus leylandii* oil was more pediculicidal than the hydrocarbon component [259]. The results so far emphasise the importance of oxygenated monoterpenoids in conferring pediculicidal activity.

### 1.13 Rationale for investigating monoterpenoid mode of action

The aim of this study is to gain more information on the relative pediculicidal and ovicidal activities of essential oil constituents and also to investigate their mechanism(s) of action.

The biological targets of the major commercial insecticides described in Section 1.6 of this chapter have been elucidated, but even though numerous studies have investigated monoterpenoid activity on various insects, no mode of action has been proven. Knowledge of the target site(s) can decide whether or not an insecticidal compound is sufficiently selective. For example, insecticidal and antifeedant furanocoumarins of various species in the Rutaceae and Umbelliferae are known to cross-link double-stranded DNA. As the fine structure of DNA is universal, this makes them potential mutagens of non-target species, which is likely to limit their commercial potential [101].

The target also determines speed of death. Although the concentration of insecticide applied affects the rapidity of action within a narrow window, the nature of the target site provides the timeframe within which the concentration 'window' occurs. For example, compounds acting on the respiratory chain or the nervous system are rapid, others, such as analogues or antagonists of the juvenile hormones and moulting hormones, affect growth and development and therefore have a delayed action. Although many hormone mimics are found in plants [111], they are perhaps not very useful in lice control as an instant cure is more desirable.

When the biological targets in one insect-insecticide interaction have been established, the application of this data to other insect species should be approached with caution. Some compounds obviously have different biological activities in different species, for example,  $\beta$ -asarone was determined to be the major active component in the insecticidal essential oil extracted from the roots of *Acorus calamus* L. (sweetflag) (Araceae). This oil is available commercially and is reputedly lethal to a range of insects, including lice. By contrast, it is a chemo-sterilant for red cotton bugs, an attractant for Mediterranean fruit flies, and a repellent towards other species [135].

Molecular-level information on an identified insecticidal target could enable a combination of binding-site modelling and synthetic chemistry to design more active and/or selective compounds. Knowledge of the target site location can help to determine the route of entry

into the insect. Changes to a chemical structure can affect its delivery to the target, as well as other determinants of activity, such as metabolism and stability, so it is important to know as much as possible about the fate of an insecticide once applied. Variation in mechanistic and/or pharmacokinetic aspects may also determine the number of insect species towards which insecticides are lethal. The majority of the commercially successful insecticides are 'broad-spectrum,' meaning they are lethal to many different insect species. Studies of monoterpenoids in the literature suggest they have rather narrow spectra of activity, and are most effective only on certain species, groups of species or even life-cycle stages. Knowledge of whether the insecticide has a broad or narrow spectrum of activity can help determine the number of species it can be used to control and its degree of threat to non-target organisms.

Most commercially exploited insecticidal molecules exert their actions via the nervous system; previous investigations of monoterpenoids suggest they are capable of rapid lethality and the symptoms of poisoning in insects and earthworms, such as knock-down and convulsions, are similar to other neurotoxic agents [70,108]. The insecticidal sesquiterpenoid picrotoxinin, the active component of picrotoxin, has been shown to inhibit GABA action when tested on an *in vitro* model of native insect GABA receptors (GABARs) [88]. This model was chosen to investigate the potential of monoterpenoids as toxicants of the insect nervous system. A model for the human GABAR was also chosen to determine whether or not the GABA-ergic activity was insect selective.

### **1.14 GABAergic neurotransmission**

The 4-carbon, non-protein amino acid GABA and the synthesising enzymes and receptors required for GABAergic neurotransmission have been described in both vertebrates [175,237] and invertebrates [119]. The major inhibitory neurotransmitters of vertebrates are GABA and glycine; in the higher centres of the mammalian CNS, GABA-mediated neurotransmission is by far the more prevalent, and occurs at about 30 % of all CNS synapses [204]. In most mammalian tissues, GABA itself is only found in traces but in brain tissue high concentrations of GABA are widespread, particularly so in the nigrostriatal system, which contains around 10  $\mu\text{mol/g}$  tissue [204], along with especially high levels of other GABAergic synaptic components. When researchers in the 1940s first described the pattern of distribution for this amino acid, a derivative of glutamic acid, it strongly implied a specific neurobiological function for GABA. Since then, GABA and GABARs have been studied in a wide range of organisms. The more extensively

characterised GABARs of vertebrates are restricted to the nervous system [268] however, in insects, as well as being present in the nervous system, they are also present at the neuromuscular junction [206,220].

In its role as a neurotransmitter, GABA is released from nerve terminals and interacts with specific binding sites on GABA receptors, which are integral membrane proteins. These GABA binding sites may be coupled to one of two types of signal transduction mechanism: In the case of metabotropic receptors, coupling is via G-proteins to  $K^+$  and  $Ca^{2+}$  channels and adenylyl cyclase, whereas ionotropic GABARs activate integral  $Cl^-$  channels. The nature of the effector system determines the end results of receptor activation and how quickly responses to the transmitter occur; ionotropic receptor-mediated events are extremely rapid, unlike the slower actions initiated by metabotropic receptors. Ionotropic,  $GABA_A$  [229] and  $GABA_C$  receptors [203], and metabotropic,  $GABA_B$  receptors [30] have been identified and classified in vertebrates; ionotropic GABARs and  $GABA_B$ -like receptors [17] have been described in invertebrates. The insect ionotropic GABAR does not readily fall into either of the mammalian ionotropic GABAR categories [119].

Although invertebrate receptors have the greater relevance in terms of this thesis far less is known about their structure and pharmacological profiles. Therefore in the following sections, accounts of mammalian GABA receptor structure, function, molecular biology and pharmacology precede those of insect GABARs.

### **1.15 Structure and function of ionotropic GABA receptors**

All ionotropic GABARs are members of the cys-loop neurotransmitter receptor superfamily. Other cys-loop superfamily members include receptors for acetylcholine (nicotinic acetylcholine receptors, nAChRs), glycine, 5-HT<sub>3</sub>, AMPA and kainate. Ionotropic receptors in this family exhibit diverse pharmacology, but their subunits share many sequence similarities and the receptors they form have similar activation/inactivation characteristics [20], for example, they are all oligomeric integral membrane proteins consisting of 5 homologous polypeptide subunits surrounding a central, transmitter-gated ion channel. All, including  $GABA_A$  subunit genes and *Rdl*, a gene encoding an ionotropic GABA receptor subunit cloned from the fruit fly, *Drosophila melanogaster*, have a conserved pair of cysteines and four regions of hydrophobic amino acids (corresponding to four transmembrane domains in the subunit) at identical positions in the gene, the second of



which translates into the putative pore-lining domain of every cys-loop receptor [131]. However, insect and vertebrate ionotropic GABARs share more structural and functional similarities than just those features demarcating the cys-loop superfamily, and are likely to have evolved from a single ancestral gene [190].

The variation among ionotropic GABARs extends further than the mammalian and insect distinction: different types of mammalian brain tissue [234] and different insect tissues [220] possess distinct GABA receptor pharmacology. Researchers therefore long-suspected a certain degree of structural heterogeneity between ionotropic GABA receptor populations, and this has been confirmed by gene cloning. GABAR subunit cloning has permitted identification of structural features responsible for the differential sensitivity to chemical agents [119,241]; known subunit combinations are heterologously expressed, and differences in pharmacology and ligand binding characteristics are related to variations in the primary amino acid sequence. Diversity within a receptor gene family enables a single neurotransmitter-binding site interaction to have a variety of consequences, not only with respect to the responses to the neurotransmitter itself, but also to those of antagonist and modulatory compounds.

The integral ion-channels of ionotropic GABARs are anion-selective; the interaction of GABA and the extracellular binding sites results, within milliseconds, in the opening (gating) of the channel and it is primarily  $\text{Cl}^-$  which passes through in physiological solutions. The direction of  $\text{Cl}^-$  flow depends on the electrochemical potential gradient (determined by the membrane potential and the transmembrane  $[\text{Cl}^-]$  gradient) for this ion,  $\text{Cl}^-$  will flow down the gradient such that  $E_m$  (the membrane potential) approaches  $E_{\text{Cl}}$  (the equilibrium potential for  $\text{Cl}^-$ ), and this direction determines the effect on the receptor-bearing cell: ionotropic GABA receptor activation can produce hyperpolarisation, depolarisation or no change in membrane potential [234]. Membrane hyperpolarisation inhibits action potential induction by increasing the degree of depolarisation necessary to reach threshold. If  $E_m$  of the post-synaptic cell is close to the equilibrium potential for  $E_{\text{Cl}}$  GABA will not effect a change in  $E_m$ , however, if the post-synaptic cell is simultaneously acted upon by an excitatory transmitter the activated GABA-gated  $\text{Cl}^-$  channels act as electrochemical shunts; the increased conductance of the membrane negates the excitatory effects, if an action potential is attained, its amplitude and conduction velocity will be lower and conduction may even be blocked. GABA-induced depolarisations occur, for example, during  $\text{GABA}_A$  receptor activity on pre-synaptic neurons and lead to a reduction in neurotransmitter release [100,234].

## 1.16 Vertebrate GABA<sub>A</sub> receptors: Molecular biology and subunit composition

The GABA<sub>A</sub> receptor is the primary receptor-transducer-effector system through which GABAergic neurons exert their action in the vertebrate CNS [100]. They have been found on the majority of neurons in the mammalian CNS and PANS (peripheral autonomic nervous system) and on various types of glia [234]. GABA<sub>A</sub> receptors occur presynaptically and postsynaptically, all serving to inhibit neuronal responsiveness (excitability) and activity in the postsynaptic neurone [240].

GABA<sub>A</sub> receptor subunit genes have been cloned from several mammalian species and the primary amino-acid sequence data analysed. The following vertebrate subunit types have so far been delineated, each of which contains one or more isoforms:  $\alpha$ (1-6),  $\beta$ (1-3),  $\gamma$ (1-3),  $\delta$ (1),  $\epsilon$ (1),  $\theta$ (1) and  $\pi$ (1) [21,25]. Homology between isoforms of different subunit types is around 30-40 %, and between isoforms of the same subunit type is at least 70 %.

Homology between identical isoforms cloned from different mammals can be greater than 90 % [190]. GABA<sub>A</sub>R subunit isoforms are encoded by members of a multigene family [21], and a further increase in diversity is conferred by the existence of splice variants of some of these genes [241]. Splice variants occur when mRNAs transcribed from the same gene undergo differential processing events, such that the variant mRNAs transcribed differ in respect of certain discrete portions of the sequence. The most prevalent isoform with splice variants is the  $\gamma$ 2 subunit, which exists as  $\gamma$ 2<sub>S (short)</sub> or  $\gamma$ 2<sub>L (long)</sub>.

In addition, the vertebrate ionotropic GABA receptor subunit genes  $\rho$ (1-3) have been identified. It is still contentious as to whether  $\rho$  subunits are a pharmacologically different subset of GABA<sub>A</sub> subunit or a different category of GABA receptor, as homomers of these subunits form receptors that are insensitive to bicuculline and the allosteric modulators that characterise GABA<sub>A</sub> receptors [203]. As a consequence of this difference in pharmacology, many authors refer to GABARs with these characteristics as GABA<sub>C</sub> receptors; this terminology will be adopted here, although it has come to light recently that at least some of the structural determinants of GABA<sub>A</sub>/ GABA<sub>C</sub> pharmacological differences are very small, some being accounted for by a single amino acid change [169].

Given the number of isoforms of GABA<sub>A</sub> subunit identified so far, there could potentially

be thousands of different receptors formed from all possible subunit combinations, however, studies of expression patterns indicate that only a fraction of the theoretically possible combinations occur *in vivo*. Studies to date suggest that the majority of GABA<sub>A</sub>Rs assemble as pentameric hetero-oligomers of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits, with a stoichiometry of  $2\alpha.1\beta.2\gamma$  (or  $2\alpha.2\beta.1\gamma$ ) [21].

### 1.17 Insect GABA receptors: Molecular biology and subunit composition

To date 6 putative ionotropic GABA receptor subunits, RDL<sub>ac</sub> [87], RDL<sub>bd</sub> [147], RDL<sub>ad</sub> [120], DRC 17-1-2 (a presumed allelic variant of RDL<sub>bd</sub>) [65], GRD [104] and LCCH3 [106] have been cloned from *D. melanogaster*. Four splice variants of the *Rdl* gene transcript encode the RDL polypeptides; *Grd* and *Lach3*, respectively, encode the other two subunits. On the basis of their high sequence identity to vertebrate ionotropic GABAR subunits, the above polypeptides were classified as *Drosophila* GABA receptor subunits, for example, the subunits encoded by *Rdl* show 30-38 % homology with vertebrate ionotropic GABA receptor subunits, a level of similarity akin to that seen between vertebrate GABA<sub>A</sub>R subunit types [119]. However, homology also exists between these insect ionotropic receptors and certain other vertebrate subtype classes, for example, RDL is equally identical to GABA<sub>A</sub>R and GlyR (glycine receptor) subunits, LCCH3 is also similar to GABA<sub>A</sub> receptor  $\beta$  subunits [106]. The heterologous expression of RDL and DRC provided further evidence that they contribute to ligand-gated Cl<sup>-</sup> channels, as the reversal potentials of the homomeric channels formed by both subunits were close to E<sub>Cl</sub> [38,88].

The *Rdl* gene is subject to alternative splicing; Two versions of exon 3 (a and b, which differ at two residues) and exon 6 (c and d, which differ at 10 residues) are known to exist, as mRNAs of all splice variants have been identified in embryonic *Drosophila* [89]. The four possible *Rdl*-encoded subunits are therefore RDL<sub>ac</sub>, RDL<sub>ad</sub>, RDL<sub>bc</sub>, RDL<sub>bd</sub>. Only RDL<sub>bc</sub> has yet to be cloned.

The occurrence of RDL homologues in other insect species has been investigated. Subunits with 85-99 % sequence homology (over their known sequences) with RDL have been isolated from a variety of insect species, including the German cockroach *Blattella germanica*, (Dictyoptera), the house fly *Musca domestica*, the yellow fever mosquito *Aedes aegypti*, *Drosophila simulans* (Diptera), the beetles *Hypothenemus hampei* and *Tribolium castaneum* (Coleoptera), and the tobacco budworm *Heliothis virescens* (Lepidoptera) [119,270].

Homologues of LCCH3 and GRD have yet to be cloned from other insects.

Subunits encoded by *D. melanogaster* *Rdl* form functional homo-oligomeric receptors in a variety of expression systems, such as *Xenopus laevis* oocytes [38,88], fall army worm (*Spodoptera frugiperda*) Sf21 cells [147], and in a *Drosophila* cell line [173]. RDL homomers show single channel properties [98] and pharmacological profiles [38] similar to those of many native GABARs studied *in situ* in insect tissue. Neither LCCH3 nor GRD form functional homo-oligomers in *Xenopus* oocytes and although, in combination with RDL, LCCH3 forms functional receptors, the pharmacological and kinetic properties do not reflect those of native GABARs on cultured *Drosophila* neurones [119]. Co-expression of GRD and RDL has not been reported.

Of the insect species listed above, all cyclodiene resistant strains investigated to date have been found to carry the same point mutation in their respective *Rdl* homologues; replacements of alanine 302 with serine (A302S), suggesting a key role for RDL-containing GABARs in insecticide action. This mutation appears to confer resistance not only to cyclodienes but also to other insecticidally-active compounds PTX (picrotoxin), lindane, TBPS (*t*-butylbicyclophosphorothionate), BIDN (3,3-bis(trifluoromethyl)bicyclo[2,2,1]heptane-2,2-dicarbonitrile) and fipronil in native and heterologously expressed *Drosophila* GABARs, as these compounds show reduced GABA-blocking abilities [23,28,39,88,113,208,280].

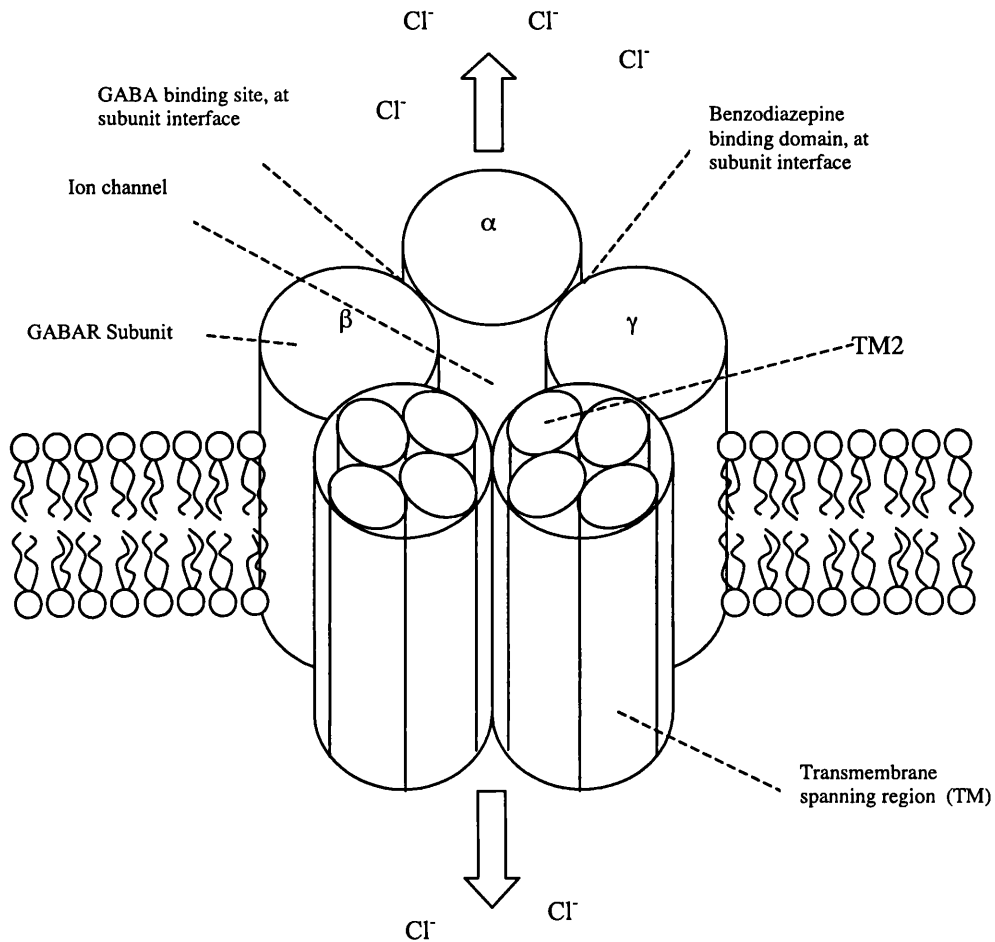
In *Drosophila*, RDL is expressed in embryonic nervous tissue [243], and RDL-like subunits are widely distributed in the nervous systems of several insect species, being particularly concentrated in neuropiles rich in synapses, as shown by antibody labelling [12,103]. Recently immunocytochemical staining of cockroach tissue revealed that the RDL homologue was especially localised in brain regions involved in processing visual, olfactory and mechanosensory inputs, and in the *corpora cardiaca*, which is involved in the regulation of neurosecretory activity [221].

Investigations on insect ionotropic GABA receptor subunits to date have been restricted to economically or scientifically important insects. So far, the existence of RDL-like subunits has not been investigated in *Pediculus* species.

### 1.18 Ionotropic GABA receptors: Allosteric proteins

A model for the topography of the GABA<sub>A</sub> receptor was constructed mainly on the basis of hydropathy profiles [234]. In this form of protein analysis, the primary sequence of the polypeptides are examined for hydrophobic and hydrophilic runs of amino acids, to enable prediction of which regions of the protein reside in the lipid bilayer and therefore which domains are extra/intracellular. Each GABA<sub>A</sub>R subunit was found to comprise 400-500 amino acid residues, 60 % lying extracellularly, 20 % in transmembrane regions and 20 % intracellularly. The various subunit types are very similar with respect to primary structure, putative secondary structure and proposed transmembrane topology [100,224,241]; Like all other ligand-gated ion channels, GABA<sub>A</sub>Rs are predicted to possess the following: i) A large extracellular N-terminal domain, containing consensus sequences for N-glycosylation and residues contributing to the ligand binding site; ii) Four membrane-spanning regions, TM1-TM4 (transmembrane domains 1-4), with each TM2 contributing to the pore lining; iii) A large, variable, intracellular hydrophilic domain or 'cytoplasmic loop' between TM3 and TM4, which contains consensus sequences for phosphorylation by protein kinases (in  $\gamma$  GABA<sub>A</sub>R subunits, this is also where alternative splicing occurs) [241]; iv) A short extracellular C-terminal portion; v) The model also predicts the presence of other, small intra- and extra-cellular loops between the TMS.

The hydropathy plot analysis of the vertebrate GABAR subunit was combined with other data on assembled GABARs, such as the identification of a 5-fold rotational symmetry of subunits around a central pore identified by electron image analysis [184], the pentameric rosette shape of GABARs as visualised by electron microscopy [33] and the high homology with other pentameric receptors such as nicotinic acetylcholine receptors and glycine receptors [241]; this led to the construction of a speculative topology of the heteromeric GABA<sub>A</sub> receptor, the pertinent features of which are shown in Figure 1.3.



**Figure 1.3 Schematic diagram of a GABA<sub>A</sub> receptor molecule**

Schematic representation of the major type of GABA<sub>A</sub> receptor thought to be expressed in adult mammalian brain, ( $\alpha$ 1)<sub>2</sub>( $\beta$ 2/3)<sub>2</sub>( $\gamma$ 2)<sub>1</sub>. The five polypeptide subunits surround a central  $\text{Cl}^-$  ion channel, each subunit consisting of 4 transmembrane (TM) domains, large extracellular and small intracellular portions. The precise arrangement of subunits and transmembrane sections is not known, however, the binding pocket for GABA exists at the interface between  $\alpha$  and  $\beta$  subunits and the residues upon which benzodiazepine pharmacology depends are at the  $\alpha$ - $\gamma$  interface. There is evidence that the TM2 hydrophilic domains of each subunit line the anion channel.

This structure allows for the binding of ligands arriving at the surface of the cell to portions of the large N-terminal domain and extracellular loops. Many of the extracellularly-located amino acids possess charged side-chains and/or the ability to form dipoles and these enable ligand receptor interactions at specific binding sites; some of these sites are likely to have evolved to interact with particular structures (molecules or ions) found *in vivo* [234] but many non-endogenous compounds, with appropriate structures, also bind to these sites. For example, the GABA<sub>A</sub>  $\beta$ -subunit is photo-affinity labelled by the agonist [<sup>3</sup>H]muscimol [40] and mutations at the N-terminus affect agonist potency of recombinant GABA<sub>A</sub> receptors [8] suggesting an extracellular agonist binding site. Furthermore, residues of the GABA<sub>A</sub>  $\alpha$ -subunit also influence agonist activity, suggesting that binding occurs at the interface between two different subunits,  $\alpha$  and  $\beta$  [236]. Recently novel determinants of agonist potency have also been defined at the N-terminus of RDL [120].

Competitive GABA antagonists such as bicuculline and SR95531, by definition, must also bind extracellularly, and GABA-like motifs have been identified in both [76,105]; it is therefore likely that at least part of the antagonist molecule interacts with exactly the same portion of the receptor as GABA. However, competitive antagonists are often larger than agonists and probably also interact with other areas around the agonist binding site; differences in these additional binding domains probably account for the fact that there are substantial differences between the competitive antagonist profiles of GABA<sub>A</sub> and RDL<sub>ac</sub>, yet their agonist profiles and the potency of GABA are similar [115].

The binding site of the non-competitive GABA antagonist picrotoxinin has been studied and several lines of evidence, including the position of the A302S mutation, suggest that the (uncharged) picrotoxinin molecule binds within the channel lumen rather than to extracellular residues [23]. Furthermore, there are a multitude of sites for allosteric modulation on ionotropic GABARs, many of these are in the extracellular portion with probably at least some at subunit interfaces [100]. Apart from regulation by external ligands binding, receptor function can also be regulated by kinases, which affect the phosphorylation state of the receptor at the intracellular face [231].

A combination of molecular biology and electrophysiological techniques have enabled identification of areas on ionotropic GABAR subunits which could be instrumental in ligand interaction: binding site models are constructed by comparing the amino acid sequences of pharmacologically different subunits and observing changes in the ligand-interaction properties of a single subunit by site-directed mutagenesis, the model can be

further developed by structure-activity analysis of radio-labelled ligands which bind to an identified site. For example, the generation of chimaeric receptors using cloned sequences for the alcohol sensitive GlyR  $\alpha 1$  subunit and the alcohol insensitive GABA  $\rho 1$  subunit enabled researchers to pinpoint areas that conferred alcohol sensitivity to GABA  $\rho 1$  subunits [169], and the nature of the convulsant binding site has been investigated using several series of structurally related compounds to construct a pharmacophore model [209 and refs within].

### **1.19 Ionotropic GABA receptors: Pharmacology**

The  $\text{Cl}^-$ -conducting activity of GABA<sub>A</sub> receptors and insect ionotropic GABARs is influenced by a diverse array of chemical structures, encompassing naturally-occurring, semi-synthetic and synthetic moieties. In the absence of pharmacological agents, ionotropic receptors usually spontaneously open and close, albeit with low frequency. For GABA<sub>A</sub> receptors, spontaneous openings are absent or rare in native and heterologously expressed recombinant receptors [100]; and the same was found recently in single-channel recordings of native *Drosophila* GABARs and heterologously expressed RDL homomers [280,281]. Increasing concentrations of GABA increase the frequency of channel opening of GABA<sub>A</sub> receptors [158], but the higher the concentration of GABA and the longer the time it is applied the more channels become desensitised and unresponsive. Receptor desensitisation is a common feature of ligand activated transduction mechanisms, and probably serves to prevent over-stimulation of the cellular processes influenced by receptor activity.

Other ligands binding to the GABA receptor affect the activity of the channel, either directly or by altering the agonist-induced response. Such effects are mediated either by ligand-induced conformational changes of protein structure, affecting gating characteristics, or by physical occlusion of either the agonist binding site or the chloride channel itself. Agonists capable of eliciting the greatest possible response (current flow) through GABARs are “full agonists,” those agonists whose maximal response falls short of the full response are “partial agonists” [see for example 129]. Some ligands bind to the agonist site but do not have intrinsic activity, and only serve to block the agonist effect, these are “competitive antagonists.” Non-competitive antagonists also reduce the efficacy of the agonists but instead of competing for the agonist binding site, they either occlude the ion channel, preventing  $\text{Cl}^-$  ion flow, or bind elsewhere and affect the conformation of the receptor such that the agonist cannot activate it to the same degree [see for example 71]. Whereas agonists preferentially bind to and stabilise the active conformation of the



receptor, inverse agonists have greater affinity for the inactive conformation, thus producing the opposite pharmacological effects to an agonist [see for example 129]. Furthermore, many “positive allosteric modulators” [100,102], agents whose binding to a separate site on the receptor serves to increase the efficacy of the agonist, have now been characterised for both vertebrate GABA<sub>A</sub> and invertebrate bicuculline-insensitive channels. Despite the convenience lent by such classification, interactions of ligands and their receptors do not always fall into a single category: for example, at vertebrate GABARs, DMCM (methyl 6,7-dimethoxy-4-ethyl- $\beta$ -carboline-3-carboxylate) inhibits GABA currents at nM concentrations but stimulates sub-maximal GABA responses at  $\mu$ M levels [230]; many GABA<sub>A</sub> general anaesthetic positive modulators, such as barbiturates, act as agonists at high concentrations [102]; and some agents act in different ways on different receptors, for example, 4'-chlordiazepam is an antagonist at GABA<sub>A</sub>Rs but potentiates at insect GABARs and RDL homomers. Table 1.1 provides an overview of GABA<sub>A</sub>, native insect and RDL receptor pharmacology.

GABA<sub>A</sub> receptors are distinguished by their sensitivity to the pthalide isoquinoline alkaloid bicuculline and are subject to regulation by numerous allosteric modulators [229]. By contrast, GABA<sub>C</sub> receptors are insensitive to both bicuculline and the GABA<sub>A</sub> modulators [201] and furthermore GABA<sub>A</sub> and GABA<sub>C</sub> receptors have distinct agonist pharmacological profiles; for example isoguvacine and ZAPA are effective at GABA<sub>A</sub>Rs but not at GABA<sub>C</sub>Rs [7,203,273].

Insect ionotropic GABARs do not fully match the pharmacology of either vertebrate class: the majority of native insect GABARs are bicuculline-insensitive [220] but, in contrast to GABA<sub>C</sub>Rs, they can be modulated by a similar range of structures to those affecting GABA<sub>A</sub>Rs [29,119,183] and are also activated by isoguvacine [219] and ZAPA [247]. Native insect GABARs are also distinct from both GABA<sub>A</sub> and GABA<sub>C</sub> receptors in terms of the relative potency and efficacy of a series of various GABA analogues [115]. Subtypes of native insect GABA receptor also appear to exist, for example, some bicuculline-sensitive insect GABARs were found in the antennal lobe of *Manduca sexta* [263], furthermore, isoguvacine and 3-APS (3-aminopropane sulphonic acid) are active agonists at several insect GABARs, but not at those of the giant interneuron GI2 from *Periplaneta americana* [220]. Variation amongst insect GABARs is not necessarily due to interspecific differences, but may depend more on the tissue from which they originate, as is the case in vertebrates. GABARs of insect muscle appear to share some pharmacological properties with those of insect neurons, but not all [222].

	Vertebrate GABA <sub>A</sub> Rs	Native Insect Ionotropic GABARs	RDL <sub>ac</sub> Homomers	Refs
<b>Agonists</b>				
GABA	Full agonist	Full agonist	Full agonist	234,206,115
Muscimol	Full agonist	Full agonist	Full agonist	234,220,115
Isoguvacine	Agonist	Agonist at some; Similar potency as on GABA <sub>A</sub>	Agonist	234,206,219, 115
3-APS	Agonist	Agonist at some; Less potent than on GABA <sub>A</sub>	Agonist; Less potent than on GABA <sub>A</sub>	206,220,115
<b>Antagonists</b>				
Bicuculline	Antagonist	Inactive at most; antagonist at a distinct subset	Inactive	229,220,219, 263,38
Picrotoxinin	Antagonist	Antagonist	Antagonist	74,220,206,38
Pitrazepin	Antagonist	Antagonist, less potent than on GABA <sub>A</sub>	n.t.	234,206,219
Dieldrin	Antagonist	Antagonist, less potent than on GABA <sub>A</sub>	n.t.	183,206
BIDN	Antagonist	Antagonist	Antagonist	209,114
γ-HCH	Antagonist	Antagonist	n.t.	181,183,206
Fipronil	Antagonist	n.t.	Antagonist	288,113
<b>Benzodiazepines</b>				
4'- chlordiazepam	Antagonist	Potentiates	Potentiates, but potency lower than on native insect receptors.	202,14,116
Flunitrazepam	Potentiates	Potentiates at some, inactive at others	Inactive	219,247,14 116
<b>β-carbolines</b>				
DMCM	Potentiates at high conc.s; inverse agonist at low	Inverse agonist	n.t.	230
3-HMC	n.t.	Inverse agonist	Inactive	16,116
<b>Barbiturates</b>				
e.g. Phenobarbitone, pentobarbitone	Potentiate; intrinsic effect at high conc.	Potentiate, but less potent than on GABA <sub>A</sub> ; no intrinsic effect.	Potentiate, but less potent than on GABA <sub>A</sub> ; no intrinsic effect.	102,213,249, 116,24,29
<b>Steroids</b>	Potentiate; intrinsic effect at high conc.	Potentiate, but less potent than on GABA <sub>A</sub> ; no intrinsic effect	Potentiate, but less potent than on GABA <sub>A</sub> ; no intrinsic effect	24,144,207, 24,29
<b>Propofol</b>	Potentiate; intrinsic effect at high conc.	n.t.	Potentiate, but less potent than on GABA <sub>A</sub> ; no intrinsic effect	3,216,217 24,29
<b>δ-HCH</b>	Potentiates	n.t.	Potentiates	181,24,29
<b>Zn<sup>2+</sup></b>	Antagonist at some	Antagonist at some	Inactive	234,
<b>Loreclezole</b>	Enhances at β2 and β3-containing Rs, less at β1	n.t.	Enhances: same potency as on GABA <sub>A</sub> β1	258,24,29

**Table 1.1 Pharmacology of vertebrate GABA<sub>A</sub> receptors, and invertebrate ionotropic GABA receptors**

Overview of the pharmacology of vertebrate GABA<sub>A</sub> receptors, native insect ionotropic GABA receptors and heterologously expressed homomeric insect GABA receptors containing the *Drosophila melanogaster* RDL<sub>ac</sub> GABA receptor subunit. Electrophysiological data only; n.t. = not tested; intrinsic effect = agonist effect when applied alone.

Delineation of insect GABA receptor pharmacological subtypes is less extensive than in vertebrates, as relatively few subunits have been cloned and successfully expressed in heterologous systems. The three insect subunit homomers that have been expressed, RDL<sub>ac</sub>, DRC 17-1-2 (RDL<sub>bd</sub>) and RDL<sub>ad</sub>, appear to differ only in their affinity for GABA, the relative potency of GABA analogues is preserved, and only very modest differences in sensitivity to selected modulators (pentobarbitone, propofol and anaesthetic steroids) were observed between RDL<sub>ac</sub> and DRC 17-1-2 [24,115,120].

Regarding the modulator pharmacology of native insect ionotropic GABARs and mammalian GABA<sub>A</sub> receptors, in general, the structural classes of allosteric modulators are the same, differences lie in the potencies and efficacies of individual compounds. The most widely researched classes of GABA-modulating molecules are outlined in Table 1.1 and are as follows:

### **Barbiturates**

Barbiturates have a wide variety of clinical effects in mammals, including sedation, reduction of anxiety, induction of sleep, and inhibition of convulsions. All of these can be attributed to their CNS-depressant actions, most of which are known to result from enhancement of GABAergic neurotransmission. For example, pentobarbitone has been studied on both native [213] and recombinant GABA<sub>A</sub> receptors of varied subunit combination [249]. In all cases it elicited three different effects consisting of potentiation, direct activation and putative channel block, each requiring a higher concentration of pentobarbitone to occur. Both RDL<sub>ac</sub> and DRC 17-1-2 homomers expressed in *Xenopus* oocytes are potentiated by pentobarbitone [24] but, in the same study, pentobarbitone was far less potent at enhancing the GABA response of the insect homomers than that of the mammalian GABA<sub>A</sub>  $\alpha_3\beta_1\gamma_{2L}$  receptor; furthermore, high concentrations of pentobarbitone had direct agonist activity at the mammalian receptor but not on the insect homomers. As a modulator, pentobarbitone has low potency on native as well as heterologously expressed insect receptors; maximum enhancement due to 100  $\mu$ M pentobarbitone applied to *Locusta migratoria* and *Schistocerca gregaria* neurones (170 % of control) was similar to the degree by which 10  $\mu$ M pentobarbitone potentiated the response to EC<sub>10</sub> GABA on RDL homooligomers [116].

### ***Steroids and propofol***

Other anaesthetics, the effects of which have been documented for various GABA<sub>A</sub> receptors and insect receptors, include propofol and the mammalian endogenous steroid 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (also known as allopregnanolone, 3 $\alpha$ -OH-DHP and 5 $\alpha$ 3 $\alpha$ ). The GABA potentiating effects of propofol have been observed in native [3] and recombinant GABA<sub>A</sub> receptors [216,217], but not native insect receptors. In a comparative study [24] propofol was found to enhance GABA action at a recombinant insect (RDL<sub>sc</sub>) and a mammalian GABA<sub>A</sub> receptor with equal potency, but the degree of potentiation was again far less for the insect receptors. Propofol also showed direct agonist action at mammalian receptors at concentrations higher than those required for potentiation, but this was not observed in homomers of RDL or those of the RDL splice variant.

5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one displayed the same pattern of potentiation and direct effect on mammalian native and recombinant receptors [24,144], coupled with only weak potentiation but no direct effect on RDL or splice variant homomers [24]. GABA responses in the cell body membrane of *P. americana* were not affected by 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one or 5 $\alpha$ -pregnan-3 $\alpha$ -ol-11, 20-dione [207].

### ***Hexachlorocyclohexanes***

In contrast to the GABA-potentiating compounds mentioned so far,  $\delta$ -HCH potentiates both recombinant GABA<sub>A</sub> and RDL [24] with a similar potency, although on the latter the maximal effect was much greater. Potentiation induced by  $\delta$ -HCH has also been documented for rat dorsal root ganglion GABA<sub>A</sub> receptors [181]. Furthermore, the loreclezole-mediated potentiation of GABA responses in RDL homomers is similar to that seen in some recombinant GABA<sub>A</sub> receptors but not others: loreclezole is three times more potent on RDL homomers as on  $\beta$ 1-containing GABA<sub>A</sub> receptors [24], but twenty-fold lower than on GABA<sub>A</sub> receptors containing  $\beta$ 2 and  $\beta$ 3 subunits [258].

### ***Benzodiazepines***

Benzodiazepines have similar clinical effects in mammals as barbiturates (they are also sedative, hypnotic and anticonvulsant). They are also anxiolytic and reduce aggression, muscle tone and coordination in mammals. Insect benzodiazepine binding sites are pharmacologically quite distinct from those of vertebrate CNS GABARs, as demonstrated initially by binding studies: the ligands Ro 5-4864 (4'-chlorodiazepam), diazepam,

flunitrazepam and clonazepam are displayed here in order of most potent to least potent at displacing the ligand [ $^3\text{H}$ ]-flunitrazepam from *P. americana* nervous tissue, but this order of potency is almost the reverse of that found in human brain [220]. Pharmacologically, differences between benzodiazepine binding sites of ionotropic GABARs are illustrated by the actions of 4'-chlorodiazepam as a non-competitive antagonist of GABA<sub>A</sub> receptors (probably at a site distinct from that of other benzodiazepine modulators) [202], and as a positive allosteric modulator of both native insect receptors [14] and recombinant RDL homomers [116], although it has reduced potency on recombinant as opposed to native insect receptors. Furthermore, flunitrazepam is an allosteric enhancer at GABA<sub>A</sub> receptors and also some native insect ionotropic GABARs [219,247], but has no effect at other native insect receptors [14,247] and RDL homomers [116]. Various  $\beta$ -carbolines act as inverse agonists of GABA<sub>A</sub> receptors at the benzodiazepine site, and have similar effects at some insect GABA receptors; 3-HMC (3-hydroxymethyl- $\beta$ -carboline), for example, partially blocked the GABA response of an identified cockroach neuron [16], however, it is apparently without effect on RDL homomers [116].

Of all the compounds that potentiate the activity of ionotropic GABARs, only one,  $\delta$ -HCH, is documented as insecticidal; all other GABAergic insecticides known to date are antagonists. Although there are certain antagonist compounds which are largely specific for vertebrate GABA<sub>A</sub>Rs, such as bicuculline, picrotoxin and the steroid derivative RU 5135 [219], insecticidal antagonists such as the cyclodienes (e.g. dieldrin) hexachlorocyclohexanes (including lindane, but not  $\delta$ -HCH) and picrotoxin, are not selective for invertebrate receptors and can even have equal or greater activity on GABA<sub>A</sub>Rs [206];  $\delta$ -HCH possesses similar potency on GABA<sub>A</sub> and insect GABARs. The avermectins are also not specifically invertebrate GABA receptor modulators, however they do exhibit low mammalian toxicity [78]. The relatively novel insecticide fipronil is, so far, the most successful in exploiting the distinct pharmacology of insect GABA receptors; although it blocks Cl<sup>-</sup> currents at both insect [38] and mammalian [288] ionotropic GABA receptors, it has much greater binding selectivity for the insect type [289].

## 1.20 Natural Products provide potential and confirmed GABA receptor ligands

The wide spectrum of structures that interact with GABA receptors encompasses both naturally-occurring and synthetic compounds. Many well-known GABA receptor ligands are native to plant and/or animal tissue and GABA itself is a significant component of the free amino acid pool in most pro- and eukaryotes. Although it is not known whether GABA performs a physiological role in plants, interestingly its synthesis is induced during stress conditions [228], and therefore could have a protective or reparatory role, or may be a precursor to compounds with such a function.

Benzodiazepines of the type used as drugs in the treatment of anxiety, sleep disturbances and seizures are found in mammalian tissue in trace amounts, although the possibilities for biological effect at the concentrations found are largely speculative [215]. The origin of these benzodiazepines is also in dispute: they may, in fact, originate from ingested microorganisms or plant material, both of which are known to synthesize them [100]; recently the production of benzodiazepines by plant tissue rather than by their associated flora was confirmed by observing the synthesis of delorazepam and temazepam from sterile plant regenerates in tissue culture [133]. The positive allosteric modulator ethanol is, of course, best known as a product of yeast fermentation. Furthermore, many endogenous steroids and steroid metabolites modulate GABA<sub>A</sub>, such as the two progesterone metabolites allopregnanolone and 5 $\alpha$ -pregnanediol, although so far no activity of insect steroids, such as ecdysone, on insect GABARs has been noted [206].

Some of the most widely used tools for studying the GABAR are naturally-occurring: muscimol, a full agonist which binds with high affinity to GABA<sub>A</sub> receptors, is found in the fungus *Amanita muscaria* (Fly Agaric); bicuculline, an antagonist used to distinguish between the two major types of ionotropic GABAR in both insects and mammals, is an isoquinoline alkaloid found in species of *Corydalis* and *Dicentra* (Fumariaceae) [70]. Picrotoxin has been isolated from plants of the moonseed family, Menispermaceae; it is a mixture of picrotin and picrotoxinin, the latter of which is GABAR-active. The picrotoxinin pharmacophore, as predicted by molecular modelling, was the basis for the successful insecticidal compound BIDN [209].

In the last few years, there have been increasing numbers of investigations of plant extracts and their constituents on GABARs, especially those derived from herbs having physiological effects reminiscent of other GABAergic compounds, such as pro-convulsant or hypnotic activities. Earlier studies commonly only explored binding activity, such as the numerous reports of flavonoids binding to the GABA<sub>A</sub>-linked benzodiazepine receptor [152,214,257]. Some studies focussed on the reversal of natural product-induced physiological effects by application of known GABAergic ligands; *Rubus brasiliensis* Martis (Rosaceae) hexanic fraction has anxiolytic, hypnotic, anti-convulsant and muscle relaxant effects in mice, all of which are reversed by pre-treatment with flumazenil [186]. Only in recent years have studies on ethnopharmacologically interesting plants become more comprehensive and included binding, electrophysiological and behavioural experiments. The majority of these studies have been carried out on small mammals and/or mammalian tissue; there are, at present, far fewer investigations of natural products, insecticidal or otherwise, at insect GABA receptors. A selection of the work on GABAergic natural products is given below and comprises plants and compounds with both convulsant and benzodiazepine-like properties.

Water hemlock, *Cicuta virosa* (Umbelliferae) is toxic to mammals, causing convulsions and respiratory paralysis. It contains several chemicals which inhibit the binding of [<sup>3</sup>H]EBOB (ethynylbicycloorthobenzoate), a GABAR antagonist, to rat brain cortex, and the efficacy of inhibition was positively correlated with toxicity [252]. One of these toxins, virol A, inhibited GABA induced Cl<sup>-</sup> flux in rat hippocampal CA1 neurones, apparently acting both at the GABA binding domain and the PTX site in the channel [253]. Anisatin, a toxic and insecticidally active component of the Sikimi plant was studied electrophysiologically by whole and single cell patch-clamp on rat dorsal root ganglion; suppression of Cl<sup>-</sup> flux was only attained during or after application of GABA, not before, and PTX attenuated anisatin-suppression of GABA-induced currents; in binding studies anisatin inhibited [<sup>3</sup>H]EBOB binding. Both lines of evidence suggest that anisatin acts at the PTX site in the channel [126]. The alkaloid ricinine, from *Ricinus communis*, was identified as having a novel mode of inducing seizures, via the benzodiazepine site rather than the via convulsant site; induction of seizures in mice was blocked by diazepam (but not phenobarbitone), and ricinin blocked [<sup>3</sup>H]flunitrazepam binding to cerebral cortex membranes [85].

St John's Wort (SJW), *Hypericum perforatum* L. (Hypericaceae), is used for many purposes in herbal medicine, such as for insomnia, and its anti-depressant activity has been proven in clinical trials. Hypericin used to be considered the only active principle. However, recent

studies of total SJW extract showed an anxiolytic activity in mammals which was blocked by pre-treatment with flumazenil; in electrophysiological studies on GABA<sub>A</sub> receptors, hypericin reduced GABA activated Cl<sup>-</sup> currents, whereas pseudohypericin, another SJW constituent, enhanced them. Pseudohypericin is therefore more likely to be responsible for the anxiolytic effect of SJW extract than hypericin [255].

Kava is an intoxicating beverage prepared from the pepper *Piper methysticum*, it is widely consumed by people native to the South Pacific island. It's most popular use in traditional medicine is as an anxiolytic, and in this respect it is reported to perform as effectively as benzodiazepines. The kavalactones (also known as kavapyrones) were reported to be the active ingredients, as four of these showed significant anaesthetic and analgesic effects. However, an apparently non-kavalactone-related GABA<sub>A</sub> binding activity from Hawaiian kava has been described in methanolic leaf extracts [77,80].

The bark from the roots and stems of various *Magnolia* species are used in Traditional Chinese Medicine to treat a variety of disorders including anxiety and nervous disturbances. Honokiol and magnolol are major constituents of this material; both of these biphenolic compounds have anxiolytic and CNS depressant effects in mammals and both were found to potently enhance [<sup>3</sup>H]muscimol binding to various mammalian CNS tissues [4,239]. As propofol, a monophenolic, also increases muscimol binding at GABARs, honokiol and magnolol may also potentiate the GABA-induced Cl<sup>-</sup> current at GABA<sub>A</sub>Rs, but this effect has yet to be studied.

Extracts of Valerian, the common name given to the crude drug made from underground organs of plants from the species *Valeriana* (Valerianaceae), are used in folklore medicine for their sedative, hypnotic, tranquilising, and anti-convulsive effects. There appears to be a dual activity with relation to their GABAR activity, as low concentrations of extracts enhance [<sup>3</sup>H]flunitrazepam binding to GABA<sub>A</sub>Rs but higher concentrations inhibit it [191].

*Ginkgo biloba* and ginseng are herbal drugs reputed to have remedial effects on various neurological disorders. Bilobalide is a sesquiterpene isolated from the leaves of *Ginkgo biloba* L.; it has anti-depressant qualities, shortens pentobarbital-induced sleeping-time in mice [34], enhances the excitability of rat CA1 pyramidal neurones and reduces the neuronal inhibitory actions of muscimol [218]. The major component of Vietnamese ginseng saponin, majonoside-RZ has a preventative effect against psychological stress induced lipid peroxidation in mouse brain, an effect that was blocked by flumazenil and



pregnenolone sulfate (a negative allosteric GABA<sub>A</sub> modulator) [278] suggesting an action via the benzodiazepine site.

The dried flowers of *Matricaria chamomilla* L. (a species of chamomile) are used in herbal preparations to provide sedative and spasmolytic effects. A study of one of its biological constituents revealed conflicting bioactivities: the flavonoid apigenin was isolated and found to act as an anxiolytic and a slight sedative in mice, but did not have anticonvulsant or myo-relaxant activities. It also displaced [<sup>3</sup>H]flunitrazepam from central benzodiazepine sites [257]. However, electrophysiological analysis revealed that apigenin reduces GABA activated Cl<sup>-</sup> currents in cultured cerebellar granule cells, and this was reversed by flumazenil, suggesting a negative modulatory activity via the GABA<sub>A</sub>R-linked benzodiazepine binding site [13].

Of course, not all GABAergic activity is via GABA receptors and studies into natural products have also addressed this. For example *Caesalpinia sappan* wood extracts have anti-convulsant properties; further investigation revealed two compounds, sappanchalcone and brazilin, both of which were found to inhibit GABA degradative enzymes [15].

### **1.21 Monoterpenoids and other volatile natural products affect the activity and ionic balance of nerve and muscle cells**

In this thesis, mechanistic studies of pediculicidal monoterpenoids focus on the phenolics, thymol, and carvacrol. Eugenol, a structurally similar insecticidally-active phenylpropanoid derived from the shikimic acid pathway, was also investigated. To date, mechanistic studies on the interaction of these compounds with insect neuronal receptors have not been carried out. However, there are various studies that examine the effects of thymol and eugenol on vertebrate neurotransmission and/or cellular ionic composition; some of the physiological effects of these compounds may be related to events at the molecular level.

Eugenol and oil of cloves are renowned for their anaesthetic qualities, especially when applied locally as analgesics. Eugenol is still widely used for this purpose in dentistry, being present in periodontal dressings and eugenates (cements containing eugenol), which are employed in potentially painful restorative dentistry. Eugenol anaesthetises mice when administered intraperitoneally [226], and suppresses the electrical activity of some nerve or neuromuscular preparations, as expected from an anaesthetic agent: eugenol elicited

reversible inhibition of the compound action potential of rat phrenic nerve [35], and also extinguished impulse transmission in the bullfrog sciatic nerve [139].

In rat dorsal root ganglion (DRG) neurons, 1 mM eugenol activated both  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  conductances [188] and, although at lower concentrations (0.1-2.5 mM) eugenol blocked  $\text{K}^+$ -induced contracture of toad skeletal muscle, higher concentrations (3-12 mM) induced contractures, probably via release of  $\text{Ca}^{2+}$  from sarcoplasmic reticulum [146]. In guinea pig heart muscle, eugenol had a negative inotropic effect and reduced resting state contractions due to inhibition of the  $\text{Ca}^{2+}$  current, probably via  $\text{Ca}^{2+}$  channel block; a conflicting effect of  $\text{K}^+$  current inhibition was also detected [227]. However, in one study involving *in vitro* and *in vivo* tests for anaesthetic action, the rat phrenic nerve hemidiaphragm preparation and rabbit conjunctival reflex, respectively, eugenol was inactive on both accounts [95]. The effects of eugenol on electrically-active cells are clearly diverse. Despite having an inhibitory action on many preparations, eugenol elicits responses in olfactory receptor neurones distributed in the nasal cavity [31]. Olfactory neurons respond to volatile compounds as part of their function in recognising potential food sources [165]. Furthermore, the enhancing action of eugenol at heterologously expressed vertebrate ion channels was investigated by injection of rat whole brain mRNA into *Xenopus* oocytes. Ionotropic receptor-mediated GABA responses achieved after injection were potentiated by eugenol and also by the monoterpenoids pinene, citronellol and citronellal [285].

Thymol is used as a stabilising agent in halothane anaesthetic preparations, but is not considered anaesthetic itself; thymol is best known as an antimicrobial component of preparations such as mouthwashes and toothpastes. The local anaesthetic effects of thymol, and other monoterpenoids, have been studied in the rat phrenic nerve hemidiaphragm preparation, where (+)/(-)-menthol, but not thymol or (-)-menthone, increased the number of stimuli needed to elicit a response, and in the rabbit conjunctival reflex test where both menthol isomers, but neither thymol nor (-)-menthone, suppressed responses [93]. However, these experiments were the same as those described in the previous paragraph in which eugenol was also found to be inactive, and therefore other tissues or experimental models may yield positive results. Other monoterpenoids that were active in these tests included terpineol and trans-anethole, inactive compounds included +/- citronellal,  $\alpha$ -terpinene and (+)-carvone [95].

The activity of thymol in releasing  $\text{Ca}^{2+}$  from intracellular stores has also been well-documented. In neurones of the snail *Helix pomatia*, thymol induces release of stored  $\text{Ca}^{2+}$

[138], and thymol is also known to release  $\text{Ca}^{2+}$  from sarcoplasmic reticulum vesicles, a property shared by menthol and also chloroform and halothane [195]. Furthermore, the dye thymol blue was shown to be a potent inhibitor of  $\text{InsP}_3$  (inositol 1,4,5 trisphosphate) binding activity [211].

Recently, the molluscicidal activity of thymol was attributed to its effects on the activity of various cellular enzymes of the snail *Lymnaea acuminata*, thymol was found to have *in vitro* and *in vivo* acetylcholinesterase-inhibiting actions in this organism [232], and, after 96 h of exposure to sub-lethal thymol concentrations, 5-hydroxytryptamine (5-HT) and dopamine (DA) levels *in vivo* were also reduced. More new research has demonstrated the direct agonist activity of thymol and six related phenolic compounds at a neuronal receptor, the rat  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub>R, expressed in HEK (human embryonic kidney) cells. At these receptors, only compounds with a phenolic -OH attached directly to the benzene ring and aliphatic substituents in ortho position with respect to the phenolic -OH were able to elicit currents [176].

Carvacrol, like thymol, has been assayed on numerous occasions for its anti-bacterial activity, but evidence for biological properties that may influence neurotransmission, or indicate the existence of a neuronal target, is rare. One study showed that both carvacrol and thymol were the main components contributing to the anti-spasmodic activity of *Origanum compactum* [254]. Again, the stimulatory actions of carvacrol on sensory nerves have been documented: carvacrol and several other monoterpenoids were shown to induce fast wave bursts of activity in the rat rhinencephalic cortex, an activity which appears to be a common feature of many compounds that are anti-feedant towards herbivores, including another monoterpenoid, eucalyptol [283].

So far, numerous studies have indicated similarities in mechanisms of action between monoterpenoids and anaesthetic compounds; many anaesthetics are also physicochemically similar to the monoterpenoids, being volatile and lipophilic. However, just as there are non-volatile anaesthetics there are also non-volatile monoterpenoids with certain anaesthetic properties: the valepotriates, isolated from Valerian, possess sedative [261] and spasmolytic activity [260], the mechanism of action is currently unknown although valepotriates bind to, and therefore may block, CNS dopamine receptors [121]. Furthermore, not all monoterpenoids have anaesthetic-like biological activities; the essential oil of wormwood has convulsant activity and also kills worms and insects, the most likely constituent responsible for its toxic effects is  $\alpha$ -thujone. Four observations establish that  $\alpha$ -thujone is a

GABAR modulator: it gives poisoning signs in mice similar to those induced by picrotoxinin, and the symptoms due to both PTX and  $\alpha$ -thujone are alleviated by diazepam and phenobarbital; *Drosophila melanogaster* flies of the *Rdl* strain (with the A302S mutation) are resistant to  $\alpha$ -thujone as well as PTX;  $\alpha$ -thujone is a competitive blocker of  $^3\text{H}$ [EBOB] binding to mouse brain membranes; most conclusively, GABA-induced,  $\text{Cl}^-$ -mediated, peak currents in rat dorsal root ganglion neurones are suppressed (reversibly) by  $\alpha$ -thujone [110]. These results suggest that vertebrate  $\text{GABA}_\text{A}$  and insect bicuculline-insensitive GABARs may be similarly susceptible to  $\alpha$ -thujone inhibition. The effects of  $\alpha$ -thujone on heterologously expressed GABARs have not yet been investigated.

### **Summary and direction**

Natural products have a firm place in the history of human louse control. Among the chemicals of this type that provide avenues for future exploitation as pediculicides, the monoterpenoids and related essential oil constituents appear promising. So far, the activity of these compounds on human lice and eggs has not been investigated extensively. Particular monoterpenoids are already known to be especially influential towards the viability of certain other insects, but the mechanism by which monoterpenoid lethality is caused is not known. Many other insecticidally active compounds have a neuronal mode of action and there is already evidence that certain monoterpenoids are active at mammalian GABA receptors.

In Chapter 2, the activity of monoterpenoids against human lice and their eggs will be explored. Later chapters will discuss a possible mode of action at insect GABA receptors.

## Chapter 2

### Monoterpenoids and essential oils as pediculicidal agents

#### 2.1 Methods

##### 2.1.1 The Orlando strain of clothing louse as a model organism for *in vitro* pediculicide bioassays

Lice are said to be the most fully parasitic of all organisms, and, as discussed in Chapter 1, this particularly applies to head lice, which must remain adjacent to host skin at all times. Maunder believes that this is why it is so difficult to culture head lice *in vitro* [160]; the high degree of adaptation to the host environment means that no artificial substitute will lead them to thrive as well; they are fragile, and highly susceptible to dehydration. Clothing lice are less fragile, as they have adapted to the temporary changes in environment that occur when garments are removed. These lice survive well in laboratory incubators, given cloth to cling to, high relative humidity (RH) and appropriate temperature (50-90 % RH, 24-31 °C). These features make clothing lice more suitable than head lice for laboratory-based *in vitro* pediculicidal assays. A major drawback to rearing human pediculi is that wild-type head and clothing lice must be fed on human blood at least once a day. In the past, variations on the theme of artificial membranes were employed to negate the necessity for contact with human skin but, unfortunately, historical versions were obviously not very reliable, as Maunder remarked that the practical difficulties associated with their use were so great that it was better to find human volunteers [160]. However, if the colony is to provide enough adults and eggs for daily insecticidal assays and still have sufficient numbers to survive, it must consist of at least several hundred insects, and to support this many lice would require an impractical number of volunteers in order to avoid sensitisation of individuals to louse bites and faeces.

Lice are normally extremely host specific, and rodent blood is actually toxic to wild-type *P. humanus*. However, a laboratory strain of *P. h. corporis*, bred by Culpeper in the 1940s, is able to feed entirely on rabbit blood, and can therefore be fed more conveniently. This, “Orlando,” strain was first raised entirely on humans, where the lice were gradually

acclimatised to being fed one large blood meal per day instead of smaller frequent feeds. They were then fed alternately on humans and rabbits until being gradually accustomed to feeding on rabbits alone. A sub-colony of the Orlando strain is maintained by MEC Ltd, Cambridge, and these lice were used for screening essential oils and terpenoids in this study [160]. Employees of MEC Ltd fed the Orlando lice once daily on anaesthetised rabbits; between feeds the lice were kept in a large glass bowl at 24-31 °C, 50-90 % RH with pieces of cotton corded cloth on which they laid eggs. The humid environment is attained by placing a large bowl of saturated saline solution, containing filter paper to act as a wick for evaporation, in the bottom of a thermostatically controlled incubator.

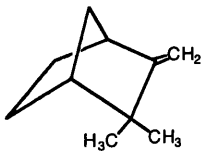
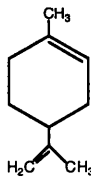
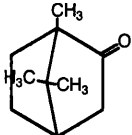
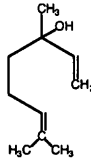
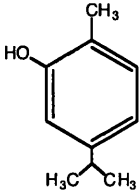
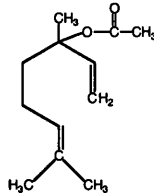
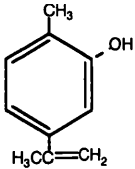
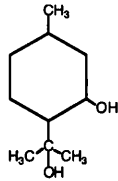
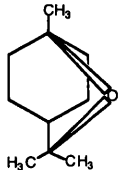
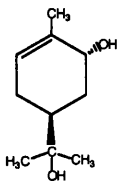
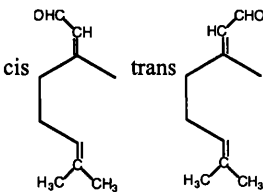
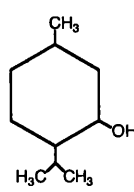
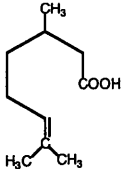
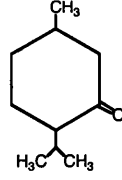
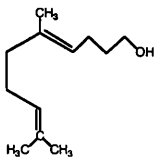
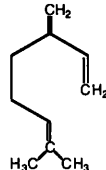
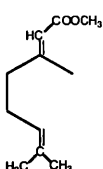
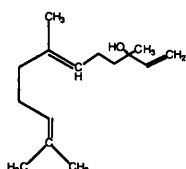
Aside from the convenience offered by Orlando strain lice with regards to feeding, it is also advantageous that the colony was established when only a few insecticides had been used to kill lice on a large scale. This means that the occurrence of resistance alleles in this population is far less likely than in lice harvested fresh from the wild. In wild populations, resistance alleles are not evenly distributed, which would necessitate collection of lice from several different areas, possibly countries, to ensure any bias due to differential susceptibility was detected. The utility of the Orlando strain as an *in vitro* pediculicide screen is illustrated by the use of both adults and eggs in numerous studies [42,43], and by Maunders' claim that it is the standard model organism across the world to represent head and clothing lice in both comparative insecticide resistance assays and in pediculicide efficacy tests. This *in vitro* model has, however, not been without its critics [125]. Details on techniques for rearing head, clothing and Orlando strain lice in captivity and references for other methods are given in [187,160].

Table 2.1 lists the monoterpenoid structures tested against lice in this study. Although the pediculicidal activity of the phenylpropanoid eugenol was not tested, Chapter 3 of this thesis demonstrates the action of monoterpenoids and eugenol at the same biological target, and it is therefore included in Table 2.1 for comparison. Included with the monoterpenoids in Table 2.1 and also in the experiments in Chapter 2 is nerolidol, strictly classified as a sesquiterpenoid, but structurally very similar to other monoterpenoids included in these tests, such as geraniol. For the purposes of this thesis, where monoterpenoids are referred to in terms of the pediculicidal and ovicidal activity, the reader should note that this refers to "monoterpenoids and nerolidol."

Terpenoids and eugenol were obtained from Sigma-Aldrich, U.K.; Lancaster Synthesis, U.K.; Acros Organics, U.K.; Fisher Scientific, U.K. or Avocado Research Chemicals, U.K..

Tea tree oil was obtained from Boots Co. PLC; eucalyptus and lemon oils were obtained from Country and Harvest, Evergreen Distribution, U.K; lavender oil was purchased from The Herbal Apothecary, U.K.

The alleged constitution of the samples of monoterpenoids and other individual essential oil constituents was examined and confirmed by thin layer chromatography (TLC).

Camphene		Limonene	
Camphor		Linalool (+/-)	
Carvacrol		Linalyl Acetate	
Carveol (-)		Menthane-3,8-diol	
Cineole		Menth-6-ene-2,8-diol	
Citral, cis/trans		Menthol (+/-)	
Citronellic acid (+/-)		Menthone (-)	
Geraniol		Myrcene	
Geranyl Acetate		*Nerolidol	



Perillaldehyde (S)-(-)		(-)-Terpinen-4-ol	
$\alpha$ -Pinene (-)		$\alpha$ -Terpineol	
$\beta$ -Pinene		Thujone (-)	
Pulegone (R)-(+)		Thymol	
$\alpha$ -Terpinene		Eugenol*	
(+)-Terpinen-4-ol			

**Table 2.1 Monoterpenoids tested for lethality against *Pediculus humanus* adults and eggs**

*\*Nerolidol, strictly a sesquiterpenoid, but structurally similar to some monoterpenoids such as geraniol, was also tested on both adult lice and eggs. For the purposes of this thesis, where monoterpenoids are referred to in terms of the pediculicidal and ovicidal activity, the reader should note that this refers to "monoterpenoids and nerolidol."*

*\*\*Eugenol, a simple phenylpropanoid, is included here for comparison. Although eugenol was not tested on *P. humanus*, its activity at a molecular target of monoterpenoids is examined in later chapters.*

### **2.1.2 The *In vitro* pediculicide bioassay**

The method used to assess the pediculicidal activity of essential oils and their constituent monoterpenoids was adapted from an established pediculicide efficacy test involving contact exposure via insecticide-impregnated filter paper [264]. Preliminary tests were carried out to determine suitable experimental parameters, such as the dilution factors of test substances and duration of exposure to lice. The finalised assay was carried out as follows:

Essential oils and terpenoids were used at 10 % (w/v or v/v), diluted in absolute ethanol, or diethyl ether if ethanol-insoluble. A volume of 0.6 ml of diluted sample was spotted onto a 9 cm diameter filter paper, held in the lower half of a 9 cm glass Petri dish. After 5 min, the liquid had spread out such that the filter paper was fully impregnated; no excess moisture was left in the dish. Impregnated papers were hung in a fume cupboard for 5 min to allow the solvent to evaporate. After this time, the papers were returned to their dishes and 10 clothing lice gently placed on top using blunt forceps. The clothing lice used were adult males and females of the Orlando strain, which had been fed 1-6 hours previously and kept at 24-31 °C, 50-90 % RH until required.

Petri dishes containing impregnated filter papers and lice were covered with glass lids and incubated at 24-31 °C, 50-90 % RH. At least two control dishes were run with each batch of insecticide assays, one containing lice on an unimpregnated filter paper, others with lice on solvent-impregnated filter papers which had also been dried for 5 min before use. Lice were observed every 15 min, by lifting the petri dish lid, and the number of fatalities recorded. In this test, death was defined by lack of movement of limbs and gut, and failure to respond when the legs were stroked with forceps. Each agent in the screen was tested 5 times in this manner. The individual essential oils and terpenoids were ranked according to their pediculicidal efficacy at 10 %, those showing no effect on lice at this dilution were unranked. Combinations of some oils were also assessed, using 0.3 ml of each of two 10 % solutions that were combined just prior to use.

Pediculicidal efficacy was expressed both as the mean ( $\pm$  one s.e.m ) % mortality for various time points and also as the  $LT_{50}$ , the time taken to kill 50 % of the lice population; log-logit transformed data was subject to linear regression analysis and the  $LT_{50}$  was then estimated from the regression line by eye as the intercept with the X-axis. Data

transformation and regression analysis were performed using the in-built equations in Graph-Pad Prism Software (Graphpad, U.K).

### ***2.1.3 Observing physiological and behavioural effects of test substances***

To gain an insight into the mechanism of action of a pharmacological agent on an organism, the first step usually involves either a casual or scientific observation of the physiological and behavioural results of application of the agent to the species in question. As lice are so small, only the grossest of symptoms can be observed; however, these may still prove useful for the purpose of grouping compounds with similar effects, in case this leads to the identification of distinct classes of test substance with different modes of action.

### ***2.1.4 Method for assaying effects of test substances on movement and behaviour of lice***

The method described in 2.1.2 was applied to a range of test substances for which observational data were to be gathered, using 5 lice per paper instead of 10. The point at which the last louse was placed on the paper was the time ( $t=0$ ) when recordings began, and these were made continuously over a period of around 1-3 hours until the lice had died or the response to the test agent remained constant over 3 time points; 2 replicates were carried out for each substance. A checklist of possible effects had been prepared beforehand from casual observation of the lice during mortality assays, and this was used as a guide when describing symptoms. When each dish was observed, the behaviour of the majority of the lice (displayed by at least 3 out of the 5) was noted; if the same symptom was not present in at least 3 lice, no score was given. The test was repeated to ensure that the results were reproducible. To facilitate analysis, the descriptive data generated was converted to table format, where each type of response seen was designated a letter of the alphabet. By comparing patterns of letters assigned, similarities between the effects of different monoterpenoids could be assessed.

### **2.1.5 In vitro assessment of ovicidal assay**

Given the poor ovicidal effect of many of the modern pediculicides, and the reputed ovicidal activity of essential oil constituents, a screen was used to distinguish whether the test substances differed in their activity on *Pediculus* eggs, and whether those that were most active on adult *Pediculus* were also most active on louse eggs. Such information could be used to determine if a single compound would suffice in a pediculicidal formulation, or whether a combination of agents would perform significantly better.

### **2.1.6 Method for assessing ovicidal activity**

The method used for testing monoterpenoid ovicidal activity was based on a method used by MEC Ltd. [42,43]. The louse eggs used were those of the Orlando strain of clothing lice. Although these lice prefer to lay eggs on tufted fabrics such as the cotton cord normally supplied, they will also lay on thick-fibred gauze if provided with no other substrate. Gauze is more suitable than cord for ovicidal assays, as the material is less absorbent, allowing the test substance to be washed off if desired. Also, the squares formed by the gauze fibres provide reference points for counting eggs and nits at the end of the test. The gauze is left in with the colony for 2-3 days to ensure a good yield of eggs, but removed at the end of day 3, as eggs laid after this time would be significantly less developed than those laid on day 1. The egg-laden gauze was removed from the incubator and immediately placed in a plastic Petri dish, packaged in bubble wrap and posted to The School of Pharmacy, where the tests were carried out on day 3 or 4. As soon as they arrived they were placed at 24-31 °C, 50-90 % RH and kept in these conditions before and after treatment with test agents.

Preliminary experiments were carried out to determine suitable test concentrations and duration of exposure to enable the relative efficacies of all agents to be assessed. In the assays, prior to immersion in the test solutions, the sheets of gauze were cut into squares of convenient size (2 cm x 2cm approx.). Terpenoids and essential oils were diluted in absolute ethanol, or diethyl ether if ethanol-insoluble, and used to fill 20 ml glass bottles. Approximately 300 eggs (200 minimum) were immersed in each test substances for 10 min. After this time, the gauze was removed, blotted with tissue paper and left for 5 min on a clean piece of tissue laid flat in a fume cupboard, on low setting, to dry off the solvent and leave the fibres of the gauze impregnated with the test substance. Eggs exposed to solvent

only were included with each batch of tests to enable correction of results with control mortality data; a control with no treatment was carried out at intervals to ensure that treatment with solvent alone continued to have no significant effect on the background mortality rate.

The eggs from individual treatments were incubated in separate glass Petri dishes at 24-31°C, 50-90 % RH until all the nymphs in the control batches had hatched and died. The eggs and nits were scored according to the identification key in Figure 2.1. For calculation of total percentage mortality, all half-hatched and unhatched nymphs were grouped together; to assess the mechanism of ovicide action, all the categories in Figure 2.1 were treated separately. In the first stage of screening, 10 % (w/v or v/v) solutions were used, the most effective terpenoids from the 10 % stage were then screened at 5 %, and the most effective from this stage were screened at 2 % and 1%. As the control (solvent alone) mortality values were regularly above 5 %, Abbott's Correction [1] was used to adjust the results of % mortality caused by test agents.

Where at least 3 replicates with corresponding control mortalities of less than 20 % (higher values render the data less reliable) were obtained for a test agent at one test concentration, the data were subjected to statistical analysis: the quantal response measure of % mortality is not directly amenable to ANalysis Of VAriance (ANOVA), as the scale of 1-100 % sets upper and lower limits for the distribution of data; if data are to follow the assumptions for the ANOVA of normal distribution and equal variance, the scale must be continuous. A common transformation for percentage values,  $\arcsin(\sqrt{Y/100})$ , where Y = percent mortality, was used with the intention of correcting the data sufficiently for analysis by ANOVA. Tukey's post-test was employed following one-way ANOVA to identify pairs of results with significantly different means; both analyses were performed by Graph-Pad Prism computer package.

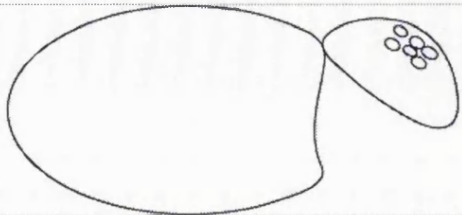
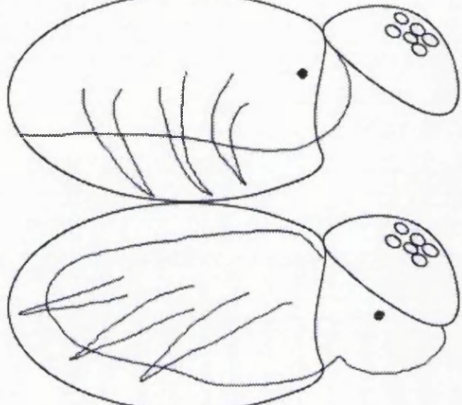

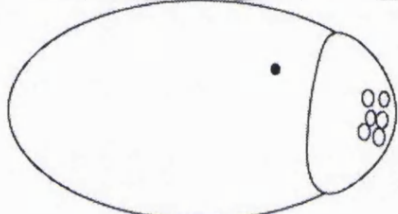
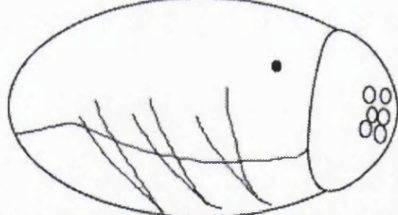
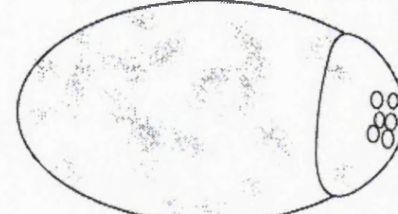
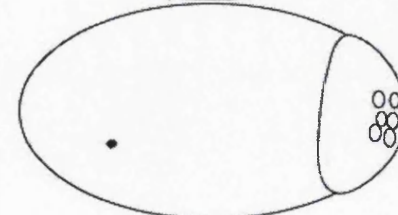
Category	Appearance	
Hatched		<p>Empty shell (nit) is transparent and shiny. Operculum open or absent. Any louse nearby has no limbs inside the shell.</p> <p>Interpretation: The agent did not kill either unhatched nymph or hatchling.</p>
Half-hatched	 	<p>Operculum partially or completely open or absent. Louse nymph either wholly or partly inside the shell.</p> <p>Interpretation: The agent killed the hatching nymph, but not the unhatched louse. This is most likely because the agent is prevented from having a true ovicidal effect as it cannot penetrate the shell, instead it persists on the fabric and kills the hatching louse when the seal around the operculum is broken.</p>
Developed	 	<p>Operculum fully closed. Correctly positioned black eyespots are present (near the cap). The formed body of the louse nymph may or may not be present within the egg: in both cases the egg appears translucent but yellow and matte in appearance.</p> <p>Interpretation: The louse embryo reached a stage of development where a functional nervous system was present, and then ceased to develop further. The agent was only active on a neuronal target.</p>
Undeveloped	 	<p>Operculum closed and egg appears matte yellow. Eyespot is either black but incorrectly positioned, red (correctly or incorrectly positioned) or absent. Often the inside of the egg has a homogeneous appearance, although a bubbly liquid interior is also common. The egg may appear shrivelled.</p> <p>Interpretation: The agent arrested development before the nervous system formed. The agent either has a non-neuronal mode of action or has neuronal and non-neuronal targets.</p>

Figure 2.1 Key to identifying the stage at which a *Pediculus humanus* egg is killed

### 2.1.7 A potential alternative pediculicide screen – The house dust mite

As explained in Section 2.1 of this chapter, it is impractical if not completely impossible to rear head lice in the laboratory. Furthermore, the use of the clothing louse as a model, even the Orlando strain, is not entirely convenient; Currently, the only accessible colony of Orlando strain lice are held at MEC Ltd, Cambridge, and to ensure that the lice are as healthy as possible before performing pediculicidal assays, the experiments must be done on site. To establish a sub-colony of this strain elsewhere would not only require special permission from MEC Ltd., but also animal care facilities and the daily availability of personnel trained to perform the animal anaesthesia and associated procedures to feed and tend to the lice. The colony would also take time to grow in number so that it was large enough support daily intensive removal for insecticidal assays. The discovery of a more convenient experimental model for *P. b. capitis* would therefore facilitate preliminary *in vitro* pediculicidal testing.

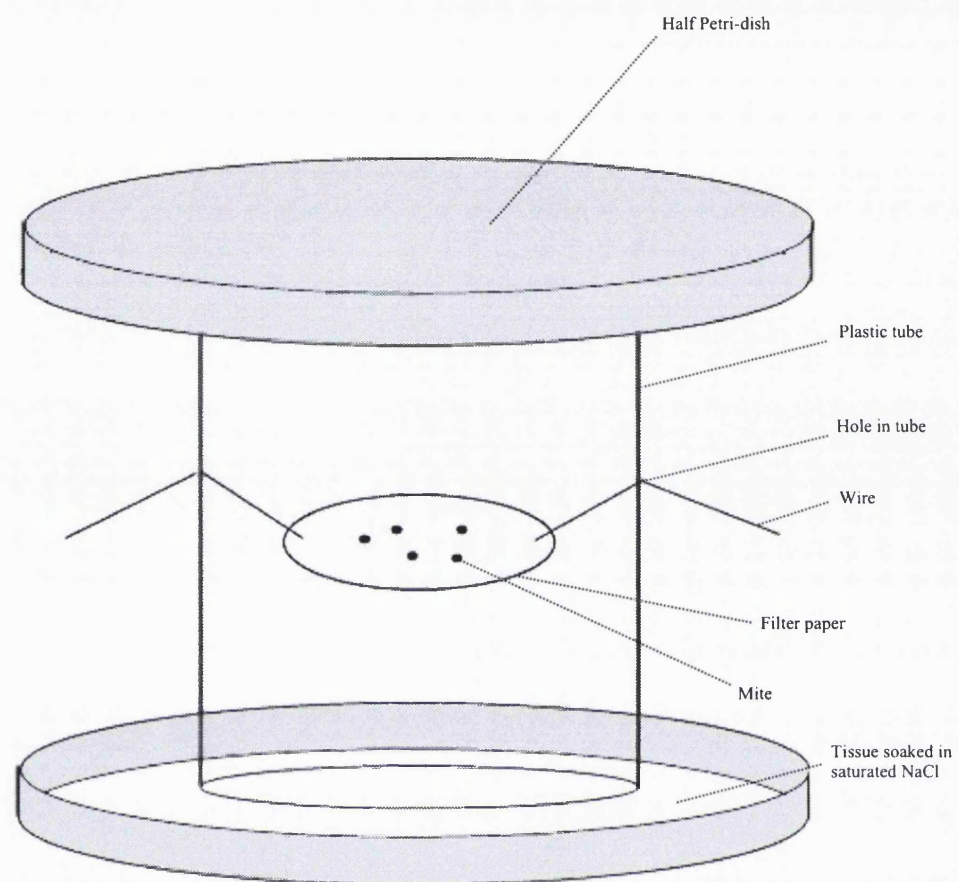
In a previous study of pediculicidal agents from oil of Leyland cypress (*Cupressus × leylandii* L.), the brine shrimp was utilised as a model organism with which to screen plant extracts and fractions [259]. Although the author reported that the brine shrimp was a suitable model with which to screen for pediculicidal agents, this assay is normally used as measure of cytotoxicity and may therefore not be ideal for the detection of louse-selective compounds; if bioactivity extends to unrelated arthropods it may be more likely to affect mammals, especially as insecticidal monoterpenoids appear to have a relatively narrow spectrum of activity on target insect species. Furthermore, it is impossible to deliver insecticides to brine shrimp in the same manner as they are delivered to lice, as brine shrimp are obligately aquatic and therefore test substances must always be applied dissolved in water. Not only are the majority of insecticides, including monoterpenoids and essential oils, mainly hydrophobic in nature, therefore making it preferable to use a non-aqueous based delivery system for screening experiments, it has also been shown that formulation is an important factor in the efficacy of an anti-lice product, therefore preliminary tests should minimise variation in excipients between the model and the species it represents.

A potentially more suitable organism to replace the Orlando strain louse is the house dust mite, *Dermatophagoides pteronyssinus*. House dust mites have recently been a subject of scientific interest, as certain allergens they produce are thought to precipitate not only

sensitisation in atopic individuals but also the consequent clinical symptoms of several closely related conditions: asthma, allergic rhinitis and atopic dermatitis [83,131]. A colony of dust mites are kept at MEC Ltd and their method of culture requires minimal time and expenditure. The following sections describe the development of a method for screening the acaricidal properties of essential oils and their constituents with a view to this species becoming a model for *P. h. capitis* in place of the Orlando strain in pediculicidal bioassays. It was speculated that the two species might respond in a similar manner to invertebrate-selective toxins, as neither is associated with plants at any stage of the life-cycle and therefore both may lack evolved species specific resilience towards plant compounds. Furthermore, neither dust mites nor the Orlando strain have been exposed purposefully to modern synthetic pesticides (it is only extremely recently that any dust-mite control products have appeared on the market); hopefully, therefore, neither will possess alleles for resistance to standard insecticides or any structurally-related compounds.

In comparison to the habitats colonised by other arthropods, the environment preferred by the house dust mite bears some resemblance to that required by the louse. *D. pteronyssinus* thrive in the warmest, most humid and least disturbed areas of the house, such as the stable climates provided deep within mattresses and pillows. They live in dust and all manner of soft furnishings and stored fabrics in houses, anywhere which traps moisture and shed skin tissue, their food source.





**Figure 2.2 A mite-chamber for testing acaricidal activity**

### **2.1.8 The house dust mite: Method of propagation**

This method of propagation of dust mite colonies was devised by MEC Ltd [52]; it promotes rapid growth and multiplication of mites and is also extremely economical on resources, with respect to the establishment and maintenance of these colonies.

Dried liver granules (Oxoid) were ground into a dust using a pestle and mortar and added to brewer's yeast powder to give a 1:1 w/w mixture. This was used to half-fill sealable plastic containers and a number of mites introduced, for example, a 1:1 v/v combination of fresh substrate and saturated colony/substrate is ideal. To facilitate gas and water exchange, parts of the plastic container should be removed and replaced with sections of porous tape, which can also be used to seal over any holes the mites can escape through. The mite colonies were incubated at 25 °C, 75 % RH and, again, saturated saline was placed in the incubator to attain high humidity.

When handling mite colonies it is important to keep allergen exposure to a minimum, and dust masks should be worn for this purpose. It is advisable to keep some of the colony in very small (50 ml max.) containers, which can then be accessed regularly during experimentation without allowing too much allergen, present in the mite faeces, to become airborne.

### **2.1.9 Testing for acaricidal activity**

A novel assay was employed in this study to investigate the acaricidal activity of essential oils against *D. pteronyssinus*. The assay was adapted from the filter-paper test mentioned previously [264], firstly to give the best chance of observing a correlation between the results of the pediculicidal and acaricidal assays, and secondly because previous assays applied to this species would not have allowed the observation of all individuals over the course of the experiment [see for example 131,223]. Dust mites are extremely small (practically invisible to the naked eye) and highly mobile; unlike lice they can scale vertical polished surfaces. The problem with any assay is containment of the creature in a localised area such that it remains in contact with the acaricide and can also be found when the assay is complete. The only way to keep the mites on the test paper is to limit their route of escape or make it so unappealing that they stay on the paper for as long as possible. Initial

experiments revealed another difficulty: although mites, even dead ones, stick to fibres on the filter paper, which has the advantage of allowing them to be observed from underneath the paper as well as from above, any slight disturbance will propel them into the air and once a mite is lost from the paper, it is unlikely to be found again. The best filter paper assay for *D. pteronyssinus* devised in this project is described below.

#### **2.1.10 The 'mite chamber' acaricidal bioassay**

A clean piece of very fine wire was used to make holes at opposite edges of a 3 cm diameter piece of filter paper, the paper was handled with forceps and contact with fingers was avoided. Sections of wire, 8-10 cm long, were then threaded through each hole a short way and the end hooked round. The filter paper with wires attached was lowered into a section of transparent tube, 5 cm tall by 5 cm diameter, until it was exactly central with respect to the length and the diameter of this tube. The free ends of the wire were threaded through holes in the tube and the ends taped to the outside. One of these pieces of apparatus, or 'mite-chamber,' was prepared for each test substance, one for a solvent-only control, and another for a chemical-free control (See Figure 2.2).

The acaricidal activity of each essential oil was assessed using a 10 % v/v solution in absolute ethanol, 0.1 ml of which was used to wet the entire filter paper, leaving no excess fluid. After waiting a few seconds to ensure that the solution had spread throughout the paper, the mite-chamber was left in a fume cupboard on low setting for 5 min to allow the solvent to evaporate. After drying, each mite chamber was placed on one half of a 9 cm Petri dish, which contained a layer of tissue paper soaked in 15 ml saturated NaCl solution in the bottom to ensure adequate humidity. Ten of the largest mites were transferred from the colony to each filter paper, using a soft paintbrush with just a few (5-10) hairs. The top of the mite chamber was covered using the other half of the Petri dish. The mites were returned to the 25 °C, 75 % RH incubator. When making observations, the location of each mite remaining on the filter paper was ascertained first using the naked eye, and then a binocular light microscope (at 25 x magnification) was used to determine whether the mite was mobile or immobile, alive or dead. The mites were assessed at 30 min for mobility and at 2 h for mortality. Mortality was defined by lack of response to stroking with a paintbrush

## 2.2 Results and discussion

### 2.2.1 Monoterpenoids as pediculicidal agents

#### *The relative pediculicidal activity of monoterpenoids*

This study demonstrates that essential oils and the monoterpenoid constituents thereof show wide variations in lethality against adult *P. b. capitis* (see Table 2.2). The  $LT_{50}$  values were estimated as described in the 'Methods' section of this Chapter, and the compounds were placed in rank order of efficacy, those with the lowest  $LT_{50}$  value having the highest efficacy (Table 2.1.1, below).

Compound	$LT_{50}$ (min)	Compound	$LT_{50}$ (min)
(+)-terpinen-4-ol	30	perillaldehyde	85
pulegone	31	geraniol	102
(-)-terpinen-4-ol	45	citral	123
thymol	45	carveol	135
$\alpha$ -terpineol	66	menthol	178
menthone	66	geranyl acetate	295
carvacrol	74	linalyl acetate	355
linalool	83		

**Table 2.1.1  $LT_{50}$  values, estimated by logit analysis, demonstrating the relative lethality of a series of monoterpenoids.**

Lice were placed on filter papers impregnated with test agent and monitored over 3h for mortality. Other agents, not listed, caused insufficient mortality to estimate this parameter.

The ranking by  $LT_{50}$ , in Table 2.1.1, is similar to those obtained at individual time points, shown in Table 2.2.

It should be noted that, of the effective compounds, perillaldehyde was of a lower percent purity than others as it was 'technical grade'. Although its position in the ranking will consequentially be less certain, as the activity could be due to a minor impurity, the indicates that either perillaldehyde or a contaminant has a high activity, and in future studies the sample could be separated into its constituent parts by phytochemical methods, and these tested individually.

### ***The relationship between monoterpenoid structure and pediculicidal activity***

This work supports previous observations that the components of essential oils that confer pediculicidal activity are oxygenated [259]. All of the compounds selected that lacked an O-containing functional group were inactive throughout the 3 h test period when applied as 10 % solutions and were as follows: camphene (bicyclic isocamphane structural type); myrcene (linear type);  $\alpha$ - and  $\beta$ -pinene (bicyclic pinane types),  $\alpha$ -terpinene and limonene (monocyclic *p*-menthane types).

Percentage mortality (% M) of lice at various timepoints (min) during exposure to the test agent shown											
30 min	% M	60 min	% M	90 min	% M	120 min	% M	150 min	% M	210 min	% M
(+)-T4ol	62 (± 13)	(+)-T4ol	100 (± 0)	(+)-T4ol	100 (± 0)	(+)-T4ol	100 (± 0)	(+)-T4ol	100 (± 0)	(+)-T4ol	100 (± 0)
Pulegone	40 (± 7)	Pulegone	78 (± 8)	(-)-T4ol	96 (± 4)	(-)-T4ol	98 (± 2)	(-)-T4ol	100 (± 0)	(-)-T4ol	100 (± 0)
Thymol	28 (± 10)	(-)-T4ol	74 (± 6)	Pulegone	92 (± 6)	Pulegone	94 (± 4)	Pulegone	100 (± 0)	Pulegone	100 (± 0)
(-)-T4ol	22 (± 2)	Thymol	62 (± 10)	Thymol	86 (± 7)	Thymol	92 (± 6)	Thymol	94 (± 6)	Thymol	98 (± 2)
Menthone	18 (± 9)	Menthone	54 (± 12)	Carvacrol	70 (± 10)	Carvacrol	90 (± 8)	Carvacrol	94 (± 6)	Carvacrol	98 (± 2)
Carvacrol	-	Carvacrol	36 (± 8)	Menthone	68 (± 15)	Perillal-	78 (± 8)	Perillal-	86 (± 6)	α-Terpineol	96 (± 4)
α-Terpineol	-	α-Terpineol	32 (± 15)	Linalool	60 (± 8)	α-Terpineol	76 (± 6)	α-Terpineol	86 (± 7)	Linalool	96 (± 2)
Citral	-	Linalool	30 (± 6)	α-Terpineol	58 (± 15)	Menthone	74 (± 15)	Linalool	86 (± 7)	Menthone	96 (± 4)
Geraniol	-	Geraniol	28 (± 10)	Perillal-	54 (± 6)	Linalool	74 (± 9)	Menthone	84 (± 12)	Perillal-	90 (± 4)
Linalool	-	Perillal-	24 (± 9)	Citral	38 (± 12)	Geraniol	64 (± 7)	Geraniol	70 (± 7)	Geraniol	82 (± 4)
Perillal-	-	Citral	16 (± 9)	Geraniol	38 (± 10)	Citral	52 (± 7)	Citral	62 (± 7)	Menthol	74 (± 11)
Carveol	-	Thujone	12 (± 6)	Carveol	30 (± 9)	Carveol	46 (± 10)	Carveol	60 (± 10)	Carveol	68 (± 12)
GerAce	-	Carveol	10 (± 4)	Thujone	18 (± 7)	Thujone	38 (± 16)	Menthol	42 (± 4)	Citral	66 (± 9)
LinAce	-	GerAce	-	Menthol	-	Menthol	14 (± 9)	Thujone	26 (± 4)	Thujone	32 (± 4)
Menthol	-	LinAce	-	GerAce	-	GerAce	10 (± 4)	GerAce	14 (± 7)	GerAce	26 (± 9)
Thujone	-	Menthol	-	LinAce	-	LinAce	-	LinAce	14 (± 7)	LinAce	24 (± 9)

Inactive: Camphene, Camphor, Cineole, Citronellic Acid, Limonene, Menth-6-ene-2,8-diol, Menthane-3,8-diol, Myrcene, Nerolidol, α-Pinene, β-Pinene, α-Terpinene,

**Table 2.2 Activity of selected monoterpenoids against the human iouse, *P. humanus***

Lice were placed on filter papers impregnated with a single monoterpenoid. Percentage mortalities (% M) caused by the test agents at set times after application (at t = 0) are shown ± one s.e.m. The test agents are shown placed in rank decreasing order of efficacy at each time point according to % M. Scores of less than 10 % (-) were discounted for the purposes of this ranking, test agents marked '-' should therefore be considered of equal rank.

Perillal- = Perillaldehyde; GerAce = Geranyl Acetate; LinAce = Linalyl Acetate

(a)

Percentage mortality (% M) of lice at various timepoints (min) during exposure to the test agent shown											
30 min	% M	60 min	% M	90 min	% M	120 min	% M	150 min	% M	210 min	% M
Tea Tree	10 (±10)	Tea Tree	34 (±17)	Tea Tree	56 (±15)	Tea Tree	74 (±12)	Tea Tree	80 (±8)	Tea Tree	90 (±8)
Lavender	-	Lavender	10 (±8)	Lavender	10 (±8)	Lavender	22 (±12)	Lavender	32 (±10)	Lavender	50 (±14)

Inactive: Lemon, Eucalyptus.

(b)

Percentage mortality (% M) of lice at various timepoints (min) during exposure to the test agent shown											
30 min	% M	60 min	% M	90 min	% M	120 min	% M	150 min	% M	210 min	% M
TT/Laven	-	TT/Eucal	22 (±9)	TT/Eucal	36 (±14)	TT/Eucal	48 (±19)	TT/Laven	64 (±7)	TT/Laven	78 (±7)
TT/Eucal	-	TT/Laven	-	TT/Laven	14 (±5)	TT/Laven	36 (±5)	TT/Eucal	50 (±19)	TT/Eucal	50 (±19)
TT/Lemon	-	TT/Lemon	-	TT/Lemon	10 (±5)	TT/Lemon	14 (±9)	TT/Lemon	22 (±7)	TT/Lemon	22 (±7)

**Table 2.3 Activity of selected essential oils and essential oil combinations on *P. humanus***

Lice were placed on essential oil impregnated filter papers.

The % mortality (% M) caused by the test agents at regular time intervals after application (at t = 0) are shown ± one s.e.m. At each time point, the test agents are shown placed in rank order of efficacy according to % M, with the highest first. Scores of less than 10 % (-) were not utilised in the ranking.

a) Relative potency of selected essential oils (0.6 ml of a 10 % solution of oil was applied to filter paper and the solvent evaporated)

b) Relative potency of combinations of oils (0.3 ml aliquots of two different 10 % dilutions were mixed prior to application to the filter paper).

TT = Tea tree; Eucal = Eucalyptus; Laven = Lavender

However, the possession of two O-containing functional groups appears to be detrimental to activity: menthane-3,8-diol and menth-6-ene-2,8-diol (saturated and unsaturated monocyclic *p*-menthane types, respectively) were both completely inactive as 10 % solutions despite the fact that, in both cases, removal of either –OH group would produce a structure almost identical to far more effective compounds such as menthol and  $\alpha$ -terpineol, and similar to terpinen-4-ol and carvacrol. It might perhaps be the decrease in lipophilicity conferred by the extra –OH group on such a small molecule that is responsible for the low activity. This theory is supported by the absence of activity for citronellic acid and the low efficacy of the esters geranyl acetate and linalyl acetate (all linear monoterpenoids). On a low molecular weight hydrocarbon, a single ester group would render the molecule less lipophilic than a single –OH group, but more lipophilic than a carboxyl group or two –OH groups. Louse cuticles are particularly waxy, therefore it may be speculated that small non-polar or slightly polar molecules might be able to enter via a trans-cuticular route, whereas more polar molecules may be prevented from doing so. The route of entry of monoterpenoids into the louse has not yet been established however, given their low molecular weight and lipophilicity, cuticular penetration is entirely feasible. The cuticle is a common route of entry for other insecticides, furthermore, lipophilic oils ('penetrants') are often added to an insecticide formulation to enhance insecticide solubility in the cuticle, this being especially important if the insect's cuticle is very waxy [78]. Veal suggested that monoterpenoids may be too lipophilic to be soluble in the haemolymph after crossing the cuticle, and therefore suggested a route of entry through the tracheae [256].

Another factor that may also influence pediculicidal activity is the bulkiness of the structure: linalool, geraniol and nerolidol are all linear types, with increasingly extended structures and decreasing efficacies. Camphor, cineole and thujone (camphane, *p*-menthane and thujane type bicyclic monoterpenoids, respectively) are more bulky than other oxygenated compounds in the plane perpendicular to the ring and, given their negligible or absent efficacy over 3 h, it appears that bicyclic structures are less effective than monocyclics in this test.

At timepoints of 60 min and above, the top 6 ranking test substances in Table 2.2 are consistently unsaturated (mono-) cyclic monoterpenoids with a *p*-menthane skeleton. At timepoints of 90 min and after, the top 4 ranking compounds in this pediculicidal assay were consistently (+)-terpinen-4-ol, (-)-terpinen-4-ol, pulegone and thymol, in that order. These top 4 compounds additionally have the following structural features in common: a

methyl group at position 1, a C attached to the ring via either a double or single bond at position 4, to which are bonded two methyl groups, and an =O or -OH functional group at position 3 or 4. There is some evidence that the particular arrangement of methyl groups may be an important determinant of activity. For example, this methyl group arrangement is present in the top 8 ranking pediculicidal terpenoids, discounting perillaldehyde (on account of impurity levels). Furthermore, the phenols thymol and carvacrol have similar pediculicidal activities consistent with their close structural similarities, yet carveol, which is identical to carvacrol except for the double bond between C7 and C8, has relatively low activity. The lower activity of carveol is surprising given that unsaturation in other parts of the molecule appears to enhance activity, for example, pulegone is more potent than menthone, suggesting that the methyl group arrangement is more important than increased unsaturation in the molecule.

A further consideration is that certain monoterpenoids may be more effective on account of their potential resilience to metabolism in the insect: for example, the tertiary alcohols terpinen-4-ol,  $\alpha$ -terpineol and linalool, the ketones menthone and pulegone, and the phenols thymol and carvacrol would all be more resistant to oxidation than the secondary alcohol menthol, the primary alcohol geraniol, and the aldehyde citral. However, this factor is likely to be secondary to those previously discussed, as the tertiary alcohols nerolidol, menthane-3,8-diol and menth-6-ene-2,8-diol are all inactive.

The activity of menthol is of particular interest, as it appears to increase sharply over time after an initial lag. Menthol is similar in many respects to the most active monoterpenoids, being monocyclic with the same pattern of methyl groups and a single -OH group, but it is distinguished by being a secondary alcohol, having no unsaturated bonds and has relatively low pediculicidal activity. The more efficacious menthone is structurally homologous with menthol except that the functional group at position 3 is a ketone instead of an alcohol. Table 2.2 shows that, although initially the two compounds have very different relative activities, the margin appears to decrease over time: at 30 min menthone is ranked 5<sup>th</sup>, at 210 min this has dropped to joint 6<sup>th</sup>; menthol has negligible activity until 120 min, where it is 14<sup>th</sup>, but by 210 min, this rank has increased to 11<sup>th</sup>. It may be hypothesised that oxidative metabolic enzymes in the insect are initially able to quench the activity of a secondary alcohol, but then become saturated over time; alternatively, the lower lipophilicity of menthol may result in a delay to sufficient amounts penetrating into the louse. Further investigation of this phenomenon is required.



The unique feature of the structure of terpinen-4-ol is that the –OH group resides at position 4, but further investigation would be needed to confirm whether or not this is the factor determining its top ranking status. It also appears that there is a difference in activity between the (+)- and (-)- optical isomers. Variations in bioactivity between isomers are an important consideration in the study of monoterpenoids, as most are optically active. It is already known that different monoterpene enantiomers can elicit different biological responses, especially at nasal olfactory receptors; for example, (+)-limonene smells of oranges, (-) limonene smells of lemons [79]. Therefore, even if not all optical isomers have been tested in a monoterpene structure-activity series, it is important to at least specify whether the test agent was the (+)-form, the (-)-form or a racemic mixture, and to identify which form(s) are present in plant essential oils.

Tea tree oil was the most effective oil in this test followed by lavender. Lemon and eucalyptus were inactive in this study (Table 2.3a). In this case, the activity of the essential oils could be predicted by first observing the activities of their major constituents, as good quality tea tree oil contains high levels of terpinen-4-ol, lavender contains mainly linalool and linalyl acetate, lemon contains mainly hydrocarbons, and the major constituents of eucalyptus are cineole and pinene [79]. In table 2.3b, the tea tree/eucalyptus combination appears to perform better than expected in comparison to the tea tree/lemon combination, and so perhaps synergy between these oils could be investigated.

The study by Downs [286] used a similar filter paper test to investigate pediculicidal activity towards freshly collected head lice from U.K. inhabitants. Tea tree oil, terpinen-4-ol, and  $\alpha$ -terpineol were all more effective against lice than  $\gamma$ -terpinene, as the experiments described in this thesis have also shown. However,  $\gamma$ -terpinene did show a much higher mortality in the Downs study, and this may have been due to slight variations in protocol; for example, the filter papers were dipped in 10 % solution rather than set amounts of solution being measured out; also, the lid was left on the Petri dish for the whole of the study, whereas it was removed at regular intervals (every 15 min) in this investigation. Perhaps the activity of  $\gamma$ -terpinene is more dependent on its activity as a vapour than that of terpinen-4-ol, as, after 2 h, some terpinen-4-ol treated lice in Downs' study were still alive. Experimental differences probably also underlie the reason that 1,8-cineole and pinene were found to be effective by Gauthier, as it was noticed when the experiments for this thesis were being carried out, that lice subjected to cineole and  $\alpha$ -pinene were highly afflicted, but did not cease movement throughout the course of this experiment; perhaps a longer incubation period would have resulted in a higher mortality for these compounds.

Although the study by Veal on individual essential oils did not discriminate between them in terms of their pediculicidal activities to a great extent; the most effective oils, giving 100 % mortality when dissolved in ethanol, were those that normally contain at least one phenolic compound in 'large' amounts consistent with the high efficacy of phenols found in this thesis (the activity profile for oils dissolved in water showed slight differences from that obtained when oils were dissolved in ethanol).

#### ***Development of the pediculicidal test for use as a screen in pharmacognosy***

The purpose of the filter paper assay, as devised by the WHO (World Health Organisation) [264], is to assess the level of resistance to insecticides in the field, presumably because of the detrimental effect of transportation on head lice viability. The filter paper test is therefore likely to have been designed primarily for convenient use in any location, whereas other *in vitro* pediculicidal assays have attempted to replicate clinical application methods, for example, sometimes lice (and eggs) are dipped in insecticide solution, washed afterwards with a mild shampoo and then rinsed with tepid water [42,43]. It may be argued that such tests might be more accurate in identifying agents that will be active in the field, as asserted by Burkhart [54], however, this may not necessarily be so if the formulation or application method changes during product development. In this test, as the solvent is completely evaporated it effectively enables monoterpenoids to be applied to lice unformulated. Different formulations and durations of exposure can influence the activity of malathion and carbaryl [42,43] and for this reason further consideration should be given as to the most suitable method by which to assess the efficacy of monoterpenoids with respect to these positive controls. One consideration of the filter paper test that particularly applies to essential oils, is that by enclosing them in Petri dishes, the agents are given the opportunity to act either by contact, vapour or both. Although the relative contributions of these two routes to pediculicidal essential oil efficacy has not been determined, in the study of *Lippia* oil on lice it was found that the toxic effects of the oil were enhanced in an enclosed test system [189], therefore it is likely that at least some volatile constituents of essential oils act partially or wholly when vaporised. The use of an enclosed system may therefore complicate comparisons with substances having only contact activity.

The next stage in determining the most pediculicidal monoterpenoids is to experiment with simplified possible essential oil/monoterpenoid formulations using various different *in vitro* methods, considering also the potential mode and duration of application of a pediculicidal product to the scalp, to see how factors such as the type of excipients and the degree of

enclosure affects the structure-activity series. These investigations will be important to determine how different experimental circumstances might affect the outcome of a pediculicidal assay, and such information may help to interpret any future results obtained *in vivo*.

The parameter used here to describe lethality, the  $LT_{50}$  (representing the time-response relationship) is a legitimate measure of insecticidal potency [5,143]. Although the majority of toxicological and insecticidal assays use  $LD_{50}$  (representing the dose-response relationship) to describe mortality; the  $LT_{50}$  was measured here because it is a useful substitute when time and insects are limited. However, if such resources become less scarce in future, the advantages of generating dose-response data should be revisited: Most insecticidal studies use the  $LD_{50}$ , and therefore comparisons between the results generated with those of other studies will be more accurate; there is also the potential to relate dose-response curves obtained at possible molecular targets to  $LD_{50}$ s obtained in toxicological studies; finally, the most effective compounds in the  $LD_{50}$  tests could be assayed for rapidity of action ( $LT_{50}$ ) by selecting several concentrations from the dose-response curve, for example,  $LD_{50}$  and  $LD_{95}$ .

It should also be noted that, although probit transformation and subsequent linear regression analysis is the standard manner by which insecticidal assay data is handled, specialised computer packages are required for probit plots and in this case the logit transformation of GraphPad Prism was successful in transforming the data to produce a linear curve.

The mortality assay employed in this study utilised the assumption that either the lice were moving and therefore alive, or immobile and unresponsive to stimuli and therefore dead. Although lack of response to stimuli has been used in other studies to define death [see for example 286], perhaps future studies should be modified to gauge recovery of responsiveness upon removal of the lice to insecticide free conditions. A different set of lice would be needed for each concentration or time point, and the insects would be incubated without disturbance for the duration of each test. At the end of the experiment, the number of lice that 'appeared to be dead' ('moribund') would be assessed as before and these lice then removed to clean filter papers and re-incubated alongside controls to observe recovery of movement over the next 24 hours. This would empower the protocol with the ability to determine if there is a minimum time of exposure or concentration required to ensure irreversibility of terpenoid action. Although lice are fragile they can, for example,

survive in water for a day via a reflex action which closes their spiracles. Some of the test compounds precipitated an almost instantaneous curtailment of motion (see Section 2.2.2 below) and, although this may reflect rapid lethality, it could also be the result of a reflex defence mechanism in lice, or even a temporary anaesthetic-type action of some terpenoids. In fact, many studies do not assay for death but 'knock-down,' the point at which the insect suffers acute neurotoxicity [143], manifested as the inability to cling to substrate or to right itself. It may, therefore, be informative to examine the effects of both lethal and sub-lethal concentrations or durations of exposure, and the points at which the effects are irreversible: Perhaps future studies could include the categories 'knocked-down' (no movement unless stimulated), reversibly paralysed (stimulated movement absent but recovers when test agent removed) and irreversibly paralysed/dead. This modification can only be included in assays if the mortality from control lice remains less than 20 % over the 24 h period (so that the data can still be corrected with Abbott's formula) [1]; the benefit of the current filter paper-Orlando strain protocol here is that no lice died in any of the control experiments, and the same control lice were still alive in the incubator the day after experimentation.

A disadvantage of the filter-paper assay is that it is not particularly suitable for the bioassay-guided fractionation of pediculicidal compounds from raw plant material: Dilute solutions of monoterpenoids and essential oils spread out evenly on filter papers very well, but preliminary experiments confirmed that crude extracts do not. In this case a dip test involving solutions, suspensions or crude formulations of extracts may be preferable or, alternatively, painting the backs of the lice with extracts could be considered. As bioassay-guided fractionation requires huge numbers of test samples to be screened, to save time and to be economical with insects, a pared-down version of the time-action curve would be particularly useful, where fewer replicates and/or concentrations were employed, reserving dose-response work for more pure fractions. As the availability of Orlando strain lice is limited, to increase the convenience and speed of the assay, an alternative organism could be used as a pediculicidal indicator (259). This possibility is discussed in further detail in Section 2.2.4.

### **2.2.2 The effect of monoterpenoids on louse physiology and behaviour**

A further advantage of the filter paper-based insecticidal activity test is that the lice can be observed through the Petri dish lid for the duration that they are in contact with the

insecticidal substance, and there are no other factors which may influence their behaviour apart from the terpenoid or oil itself, such as the lice being wet from immersion in insecticide. The collated results from the observational study are shown in Table 2.4, with the key below. The purpose of this study was to report the occurrence of different physiological effects of monoterpenoids, to assess the value of the results of this preliminary work, and to discuss whether such data should be recorded in tandem with mortality, or whether separate observations would be more appropriate.

Experiments that report gross morphological, behavioural or physiological responses of target organisms to drugs can be problematic in their design, as the parameters of interest are often difficult or impossible to quantify in a wholly objective manner, and the resulting subjective element in their assessment leaves the experiment open to scientific error. For example, in humans, a change in blood pressure can be ascertained objectively, by its value in mm Hg, but the effect of a chemical on a skin condition such as eczema is not so straightforward; as there are multiple interrelated aspects to consider, such as the size of the inflamed area, the severity of inflammation, and the appearance of the lesions. The problem can partially be solved by careful experimental design; the severity of an eczema lesion could be judged on a scale of 1 to 5, or relative to those lesions normally experienced. The patient, or better still an independent, trained observer, could make the assessment, and this would go some way to quantifying, or at least scientifically describing, the effects, but the decision will still be based on subjective judgement. An improvement for future experiments would be to take a video recording of the lice throughout the duration of test substance exposure; at least then the diagnoses made by the primary researcher could be scrutinised at any time by a second party, although analysis of the data would still present a problem.

Test Agent	Response profiles							
	Response categories (1-8) are shown with the symptom (letters A-X) elicited by the test agent displayed below. Refer to the key to this figure for symptom definitions.							
Test Agent	1	2	3	4	5	6	7	8
Control	B	C	H	J	M	R	U	W
Camphor	A	D	I	K	P	T	U	W
Carveol	A	F	H	J	O	Q	U	X
Carvacrol	A	F	H	J	O	Q	U	X
Cineole	A	D	I	K	P	T	U	W
Citral	A	E	I	J	O	R	U	W
Geraniol	B	E	I	J	O	S	V	W
Linalool	A	F	I	L	N	S	V	W
Menthol	B	E	I	J	N	S	U	W
Menthone	A	D	I	K	P	T	U	W
Perillaldehyde	B	E	I	L	N	R	U	W
Pulegone	B	F	H	J	O	S	U	W
(+)-Terpinen-4-ol	B	D	H	L	N	S	V	W
(-)-Terpinen-4-ol	B	G	H	J	O	R	U	W
$\alpha$ -Terpineol	B	F	H	I	O	S	U	W
Thujone	A	E	I	J	O	var	U	X
Thymol	A	F	H	J	O	Q	U	X

**Table 2.4 Physiological and behavioural responses of the human louse, *P. humanus*, to monoterpenoids**

Lice were observed during exposure to various monoterpenoid-impregnated filter papers. The symptoms displayed by the majority of lice were recorded and transferred to table format using the key on the following page.

## Key to Table 2.4

### Behavioural response to test substance:

(1) In the initial stages of exposure, the lice visit the edge of the Petri dish:

A - more frequently than control lice    B - with the same frequency or less

### Movement:

(2) During exposure, the rate of walking and/or limb movement was:

C - similar to control lice                      D - initially faster, then became gradually slower.

E - initially no different, then became gradually slower.

F - instantly slower, but increased with mechanical stimulation.

G - instantly slower, and was unchanged by stimulation.

(3) Ability to perform co-ordinated movement:

H - Movement was either normal, or featured only minimal mal-coordination.

I - Movements were distinctly mal-coordinated, and included staggering, non-directional perambulation and falling-over.

(4) Body movement along antero-posterior axis:

J - normal, with neither of the attributes K or L.                      K - the lice became rigid

L - arching; exaggerated movement along the antero-posterior axis

(5) Limb movements when inverted:

M - normal                      N - twitching; erratic jerking movements, may also be accompanied by flailing motion.

O - flailing; slower constant limb movements no twitching.                      P - curling; limbs bent inwards in a curling motion.

### Dehydration:

(6) The abdomen was:

Q - bloated in appearance.                      R - normal; was turgid but distensible.

S - displaying loss of turgor.                      T - shrivelled & hard

Var - turgidity varied between individuals.

### Gut activity:

(7) U - normal, or not discernibly abnormal.                      V - distinctly increased.

### Proportion of lice with ruptured guts at death:

(8) W - same as control                      X - greater than control.

During initial insecticidal activity studies, it was quite obvious that lice exposed to different monoterpenoids behaved differently and it was speculated whether such diverse effects might reflect different modes of action. However, breaking down the overall physiological effect into defined symptoms, so that compounds could be classified on the basis of the spectrum of symptoms they elicited, was not straightforward. General problems encountered included the limited duration of available observation period: many of the monoterpenoids rapidly incapacitated lice, so the time-frame in which the initial symptoms could be observed was often short and contained a rapid progression of overlapping physiological effects. Furthermore, although the response shown by the majority of lice was recorded, there was some evidence of symptom variability within individual dishes of insects; typically, one louse would behave differently from all the rest. In the case of subtle physiological effects, it can be difficult for the observer to decide that what is being observed is outside the range of normal behaviour. It should also be considered that certain responses to monoterpenoids may not be physiological indicators of a certain mechanism of action but merely general symptoms of distress, which will complicate mechanistic analyses.

Although the main purpose of using this observational method is to ascertain information on the biological effects of monoterpenoids on lice, with the expectation that compounds eliciting the same symptoms may have identical or related modes of action, it can only be speculated as to what the biological targets are; effects occurring at the level of the insect may not be directly related to the mechanism by which progression to death is elicited.

#### ***The symptoms of monoterpene toxicity, and discussion of the chosen parameters***

In this study, the terpenoids camphor, carvacrol, citral, linalyl acetate, menthone and thymol cause the lice to congregate around the edge of the dish during the initial period of exposure, probably because the insects were repelled by these test substances and could presumably sense fresh air entering the dish at the perimeter. Repellent activity of terpenoids is not necessarily related to toxicity, and is probably more effectively observed at a concentration which is sub-lethal (perhaps  $LD_{5-20}$ ), or at least which does not incapacitate the lice immediately, so directional movement is allowed. The apparatus should also be modified so that the lice are able to escape the test environment, then the parameter could be assessed quantitatively, i.e. the number moving to the terpene-free environment over time would be related to the degree of repellency of the compound.



Distinguishing different types of movement in response to terpenoids was of partial success in this study: it was envisaged that a compound with a neuronal mechanism of action may either be stimulatory or inhibitory in nature, and therefore either increase or decrease louse activity respectively. Observing rapid immobilisation of lice is straightforward, as this behaviour starkly contrasts with the constant activity of control lice. Estimating the number of lice that were hyperactive was not so simple: Many terpenoids induced some form of mal-coordination, such as walking in a 'staggering' fashion and inability of lice to right themselves. In this case the nature of the movements was so different from control lice that it was difficult to decide if the speed of those movements was faster or slower. Furthermore, effects such as mal-coordination or jerky movements of limbs varied between compounds, being subtle for some terpenoids, but more exaggerated for others; whether these were different degrees of the same response, as caused by differentially potent compounds, or completely distinct effects could not be ascertained. Another complication was that sometimes lice appeared to show more than one type of movement in a category, for example, both twitching and flailing limbs might be seen at different times in response to the same terpene, making scoring difficult, or impossible in the case of thujone.

The effect of terpenoids on gut activity was also not clear-cut, mainly because gut motility of control lice could be quite erratic. A more satisfactory parameter was the proportion of lice with a burst gut, as this feature was very distinctive (the abdomen turning bright red), and rare in control lice. However, it was not possible to score for the presence of ruptured guts in 'shrivelled' lice, as their abdomens became very small and dark coloured.

The results obtained thus far suggest that different terpenoids elicit different responses in lice. With the current methodology, some of these parameters are more reliable than others for describing the differences in physiology observed. An assessment of the most distinctive effects of terpenoids on louse physiology, as seen in this study, reveals that there are at least three bioactivity groups:

Certain terpenoids appear to cause a profound shrivelling effect in lice (see Table 2.4); although other terpenoids appeared to influence abdomen volume, either resulting in bloating or loss of turgidity, these states were less easy to distinguish from the variation in control lice because variation in both control and test groups meant a degree of overlap. Shrivelled lice were the major type found in the camphor, cineole and menthone-containing dishes. It has been suggested before that monoterpenoids, such as those present

in Suleo-M (a formulation containing malathion and terpenoidal fragrance compounds), might affect the water balance of lice [52]. It is conceivable that a large increase in the rate at which the louse loses moisture, for example, by alterations in metabolism, cuticle permeability or the properties of water-balance mechanisms, such as diuretic hormone secretion, could result in this profound dessicatory activity. Furthermore, other symptoms induced by these compounds were similar (see Table 2.4) and all three induced highly exaggerated writhing with death occurring a relatively long time after the symptoms first appeared. A characteristic curling limb movement was also noted and, although this may be a direct effect of the compounds in question, it could be a secondary effect; perhaps dehydration of insect tissue surrounded by rigid exoskeleton may have resulted in distorted appendage movement, due to loss of internal hydrostatic pressure.

Another, particularly marked effect was rapid immobilisation: Many compounds, such as the alcohols, geraniol, linalool and menthol, induced a period of mal-coordinated activity in lice before locomotion slowed and eventually stopped. Thymol, carvacrol, linalool, carveol,  $\alpha$ -terpineol, pulegone and (-)-terpinen-4-ol, however, appeared to induce a sudden decrease in voluntary locomotory activity, although in most cases the lice remained alive for some time, as touching with forceps was prone to elicit movement. The two immobilising compounds that were most similar in terms of their other physiological effects were thymol and carvacrol, the two phenolics. Swollen abdomens and burst guts were characteristic of lice exposed to these compounds.

The remainder of the compounds showed neither rapid immobilisation nor shrivelling effects, although other interesting physiological effects were noted. Some of these, such as mal-coordination and twitching, were reminiscent of the previously reported effects of neuroactive insecticides, such as pyrethroids. There were differences in the symptoms induced by these monoterpenoids, but the assay requires further development to clearly distinguish other bioactivity groups.

#### ***Suggested revisions to the observational assay***

The observational study could prove to be even more informative with further development. For example, the scoring system could be revised to allow changes in symptoms over time to be compared. The symptoms assayed could also be expanded, for example, lice exposed to  $\alpha$ -thujone were often seen to expel gut contents at a far more rapid rate than other lice, and also female lice appeared to be more afflicted by thujone

than males, these effects were not anticipated in the first study. The study could also be made more quantitative by scoring for traits at the level of the individual louse, and the data summarised. Because of the small size of lice it will probably be wise to only attempt to score discontinuous data, i.e. the presence or absence of a trait, rather than its degree or severity. Categorisation of the symptoms induced in lice could be further aided by observation of the effects of standard insecticides, or other noxious chemicals with identified biological targets, for comparison. It may also prove useful to observe these effects in larger insects first. To distinguish between specific physiological effects and general signs of distress, perhaps a non-specific, or non-neuronally active control such as a dip in olive oil or kerosene, could be included for comparison.

The observation of lice movement and other physiological events would be facilitated if an equipotent concentration of each terpenoid was used, for example,  $LC_{30}$ , ensuring that this was low enough to observe symptoms over a period of at least 1h, before death occurred. This would hopefully reduce differences in the ferocity of effects due to differential potency of the compounds.

Electron microscopy has often been utilised to aid the examination of louse structure. This technique might be useful to confirm changes in gross morphology of the louse cuticle, such as the surface texture, caused by deleterious substances. However, this technique should be approached with caution. Although invaluable for its ability to resolve fine structure, the preparation of specimens for electron microscopy is prone to producing a range of visual artefacts [194] and the surfaces viewed by scanning electron microscopy are particularly liable to become distorted during this process if their shape is dependent on hydrostatic pressure. Because lice have soft distensible abdomens designed to fill with blood, their shape may be even less faithfully reproduced by this technique than that of other insects. Consultation with an insect-imaging expert would be wise before this technique is used in observational studies of pediculicide action, and multiple replicates of control and treated insects should be analysed.

In the future, further investigation is needed into how pediculicide action at the molecular level translates into the physiological response at the level of the whole louse. Although the use of standard insecticidal compounds with pre-determined mechanism(s) of action may be informative in this respect, there are likely to be some novel effects of monoterpenoids or other essential oil constituents for which previously identified equivalents cannot be found.

### 2.2.3 Ovicidal Activity

#### *Ovicidal activity of monoterpenoids*

The results obtained in this study suggest that monoterpenoids show vast differences in potency towards *P. humanus* eggs. The method employed to evaluate lethality served a dual purpose in that it also provided information regarding the mechanism of action of the ovicidal substance. The following discussion will examine the results obtained so far, and also propose improvements to increase efficiency in the future.

#### *The potential of monoterpenoids as ovicidal agents*

The method for determining ovicidal activity described in section 2.1.6 was used to assess a selected array of essential oil constituents. A preliminary screen at 10 % w/v (solids) or v/v (liquids) allowed the test agents to be classified into two groups, one high activity, where the total mortality was 82 % or above, one low activity, giving mortalities of 34 % and below. As there was a clear demarcation between the high activity and the low activity group i.e. none of the compounds gave a (mean) mortality result in the 35 % to 81 % range, the number of replicates were kept to a minimum ( $n = 1-3$ , see Figure 2.3 (a)) which was important as the supply of eggs was limited. However, the low number of replicates means that these results should only be used as preliminary findings; they cannot be analysed statistically.

The compounds from the high activity group were then screened at 5 %, in addition to one other agent, geraniol (See Figure 2.3 (b)). To increase the accuracy of the mean percentage mortality estimate at this concentration, the number of replicates was increased so that  $n = 2-5$ . From this screen, 6 test agents with the highest mean mortalities of 89 % and above were selected for testing at two lower concentrations, those rejected gave mean mortalities of 70 % and below. The selected agents were then tested at 2 % and 1 % w/v or v/v (See Figure 2.3 (c) and (d)).

The data in Figure 2.3 suggest that there are some similarities between the results obtained for toxicity against louse adults and eggs: mono-oxygenated monocyclic terpenoids performed relatively well in the preliminary ovicidal screen at 10 %, whereas there was either no activity (cineole,  $\alpha$ -pinene,  $\alpha$ -terpinene) or low activity (limonene, menth-6-ene-2,8-diol,  $\beta$ -pinene) from the non O-containing terpenoids, the mono-oxygenated bicyclic

terpenoid or the monocyclic terpenoid with 2 O-containing functional groups. Linalyl acetate again showed low activity in comparison to the alcohols. Notable differences between the results of the pediculicidal and ovicidal assays include high activities from citronellic acid and nerolidol in the ovicidal assay, which were completely inactive in the pediculicidal study, and also lack of activity of the relatively highly pediculicidal menthone. At present these are only preliminary observations, as the data for terpenoids at 10 % suffers from insufficient replication and high control mortality. At 5 %, lower control mortalities, most likely resulting from various attempts to decrease fluctuations in the incubation environment, were attained and the data for citronellic acid, linalool, menthol and (-)-terpinen-4-ol contained sufficient replicates where the control mortality was 20 % or less to be suitable for statistical analysis. Despite large differences in mean mortality for these compounds, when the data was transformed and analysed by one-way ANOVA, followed by a Tukey post-test, no difference in the mean percentage mortalities were found for these compounds. In the light of the large s.e.m.s found for this data, it would seem appropriate to perform further replicates in an attempt to decrease the variance and to subject the data to a further ANOVA before concluding that there is no difference in % mortalities of these compounds at 5 %. For comparison, a non-parametric t-test, Welch's t-test, was performed on each untransformed pair of data sets. At the  $p=0.05$  level, a significant difference was found between the mean % mortality results for linalool and menthol, tested at 5 %. Although analysis of more than two treatments by multiple t-tests rather than ANOVA is not recommended, these results may indicate that the equation used to transform the data to make it suitable for the parametric one-way ANOVA test was not entirely successful; if further replicates are obtained, the distribution of the variances before and after transformation could be examined to confirm the suitability of the chosen corrective equation.

Statistical treatment of the results at 1 % by transformation, followed by ANOVA and Tukey post-test, appeared to be more successful. This test revealed significant differences (at  $p = 0.05$  level) between the mean % mortalities of certain pairs of compounds: nerolidol and menthol/carveol/ $\alpha$ -terpineol; geraniol and menthol/ $\alpha$ -terpineol; thymol and menthol/ $\alpha$ -terpineol, and these results agree with the graphical representation of the data in Figure 2.3.

Considering primarily the results of this ANOVA, and secondarily the graphed results (mean and s.e.m.) in Figure 2.3, a ranking for monoterpenoid lethality towards *P.humanus* eggs was produced, with the compounds being grouped according to the mean and

standard error of the resulting mortalities. Nerolidol, thymol and geraniol were the most effective ovicidal terpenoids in this screen. This data, from all the concentrations tested, was used to construct a ranking table of preliminary structure-activity relations; different groups having performed differentially at the various concentrations tested.

The ranking is as follows, with groups 1-4 listed in order of decreasing ovicidal potency. Top ranking compounds are shown next to mean percentage mortality  $\pm$  one s.e.m when tested at 1 % w/v or v/v :

- 1 - nerolidol,  $85 \pm 13$  %
- 2 - thymol,  $59 \pm 20$  % geraniol,  $56 \pm 12$  %
- 3 - carveol,  $25 \pm 14$  %
- 4 - menthol,  $2.6 \pm 2.6$  %,  $\alpha$ -terpineol,  $1 \pm 1$  %.

On the basis of the preliminary screen at 10 %, the following test compounds have tentatively been assigned joint 5<sup>th</sup> and joint 6<sup>th</sup> rankings, however, at present there are insufficient replicates to confirm these positions:

- 5- citral, citronellic acid, linalool, (+)-terpinen-4-ol, (-)-terpinen-4-ol.
- 6- cineole,  $\alpha$ -pinene,  $\alpha$ -terpinene, limonene, menth-6-ene-2,8-diol,  $\beta$ -pinene, linalyl acetate, menthone.

The evidence for the relative positions of the top 6 compounds is more firm than for the remainder, and these results alone show that there are differences between the structure-activity relations of monoterpenoids on louse adults and eggs, using the methods described in this study. For example, some straight chain structures such as nerolidol and geraniol have equal or higher ovicidal activity than cyclics or phenolics, whereas they have comparatively low pediculicidal activity. Furthermore the top ranking compounds are two tertiary alcohols, one secondary alcohol and one primary alcohol and two phenolics, if metabolic protection from toxicity via oxidation is a determinant in monoterpene activity, it may be that it is less active in the embryo.

The louse egg cuticle is hydrophobic and almost impenetrable, gaseous exchange can only occur via the small group of aeropyles in the operculum. Insecticides entering via these pores require careful formulation to do so: the narrow entrance to the pore creates surface tension to prevent penetration of aqueous solutions, and the hydrophilic inner structure of the pore lining hinders absorption of organic solvent; the physicochemical properties of insecticides are therefore of the utmost importance. It may be speculated that surface

tension-lowering ability is the reason why geraniol and nerolidol perform better in the ovicidal assay than the pediculicidal assay; they may be acting as weak surfactants by the virtue of a single –OH molecule at or near one end of a long aliphatic portion, and therefore may be able to penetrate the pores more effectively. As the phenolics are less likely to have this property, it could be considered whether their route of entry is actually different from that of the straight chain alcohols; perhaps as they are more hydrophobic, small, flat molecules, they may be able to cross the shell cuticle. Further analysis of structure-activity relationships is clearly required, but it may reveal that there are, in fact, two groups of ovicidally-active monoterpenoids with different routes of entry.

Regarding the differential ranking between the pediculicidal and ovicidal assays, one consideration is that it may perhaps have been the result of the differences in methodology between the two tests. In the pediculicidal test, the lice effectively did not contact ethanol because it was evaporated before they were placed in contact with the test papers, the eggs were exposed to it for 10 min. Although ethanol alone does not significantly affect hatching when only applied for 10 min, it may be that the presence of certain monoterpenoids, which are used in some drug formulations to improve penetration of the active components through membranous barriers, may actually be enabling ethanol to enter the egg. Alternatively, ethanol may be enhancing the activity of particular monoterpenoids over others. In future tests where comparisons of pediculicidal and ovicidal activity are required, the methodologies should be modified such that the vehicle, mode of application, and all other associated procedures are as similar as possible.

The results obtained here agree with the results of Veal *et al* [256], who showed that the relative proportions of oxygenated compounds and hydrocarbons in an essential oil were crucial to determining ovicidal activity *in vitro*. Rosemary and pine were the only oils not to cause any ovicidal mortality when tested individually [256]; rosemary contains primarily hydrocarbons and bicyclic monoterpenoids, and both types of compound were ranked in the least ovicidal group in this study; the composition of pine oil depends on the species, but hydrocarbons are sometimes the primary constituents [79]. The Veal *et al* study also provides further proof that the terpenoid components of ‘Suleo’ insecticides are responsible for their high ovicidal activities, as the constituent terpineol was among the most ovicidal in this test, and limonene also appeared to show moderate activity.

This study indicates the potential for some monoterpenoids to be included in formulations as ovicidal agents. Provided that the environmental conditions remain favourable, viable

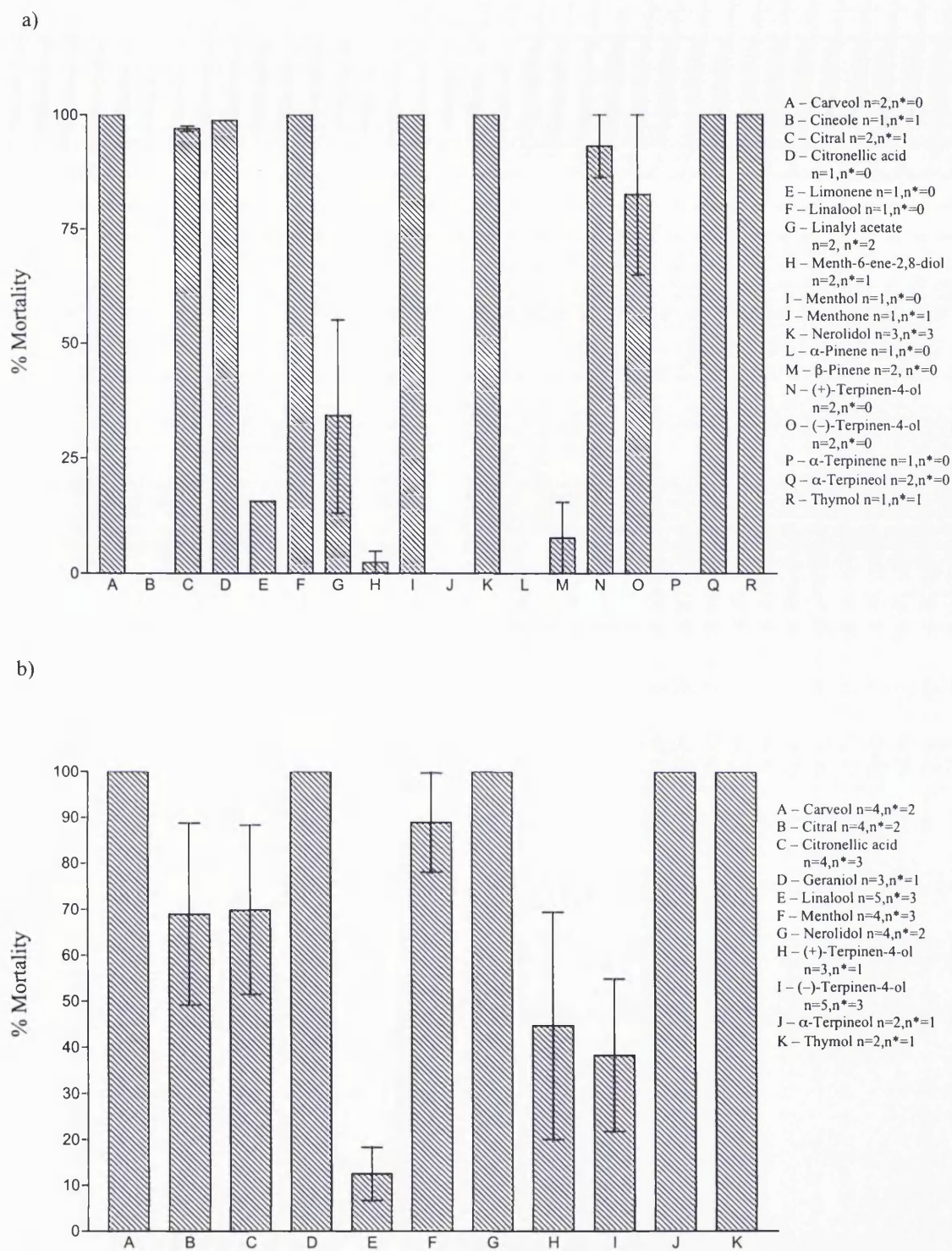
louse eggs are very resilient entities in terms of their ability to withstand exposure to chemicals; some preliminary studies with ethanol revealed that the mortality rate was not greatly affected by incubations in ethanol of up to 2 hours. Conversely, some monoterpenoids with a similar structure to ethanol in that they are small, O-containing volatile agents, are highly active against eggs in a very short space of time. The results of this work show that a 2 % solution of thymol could achieve a near 100 % kill in 10 min, and preliminary studies suggest that a 10 % solution can achieve 100 % mortality with just a 10 sec incubation time. It has not yet been determined whether these short application times will be as successful *in vivo* and clinical studies will therefore be necessary. Although these concentrations of terpenoids are quite high for therapeutic agents, provided that the safety margins are sufficient, topical application of an insecticidal substance at 1 – 10 % may be feasible. Any necessary reduction in concentration could probably be rectified by extending the application time; the current recommendation for most pediculicidal products sold in the U.K. is to leave them on for 12 h, therefore exposure times for essential oil constituents longer than 10 min may be permissible.

#### ***Developing a strategy for assessing ovicidal activity***

Unlike other biochemical assays or knock-down lethality assays on adult insects, assessment of ovicidal activity towards *P. humanus* eggs is inherently slow, as it takes around 2 weeks for them to hatch. With the added complication of a variable natural mortality rate, making it necessary to discard some of the batches if the 20 % threshold for control mortality is to be adhered to, assessment of ovicidal activity is consequently a very low-throughput screening process. It is therefore especially important in this case to devise two different strategies for assessing the ovicidal potential of a chemical; a quick 'look-see' test could be implicated to ascertain that the compound is active above a certain threshold for preliminary work or bioassay-guided fractionation work. A lengthy screen, involving a greater number of eggs, multiple replicates, and strict rejection where the control mortality is greater than 20 %, could be employed for work of publication standard, and full dose-response and/or time-action data of this nature could be considered for particularly active compounds. If percent mortality alone is to be assessed accurately in a batch of eggs, rather than the percentage contributions of all possible stages at which death did or did not occur, the number of eggs could be reduced (possibly to 50-100) which would also make better use of time and resources. Mechanistic studies could then be reserved for the most active compounds.

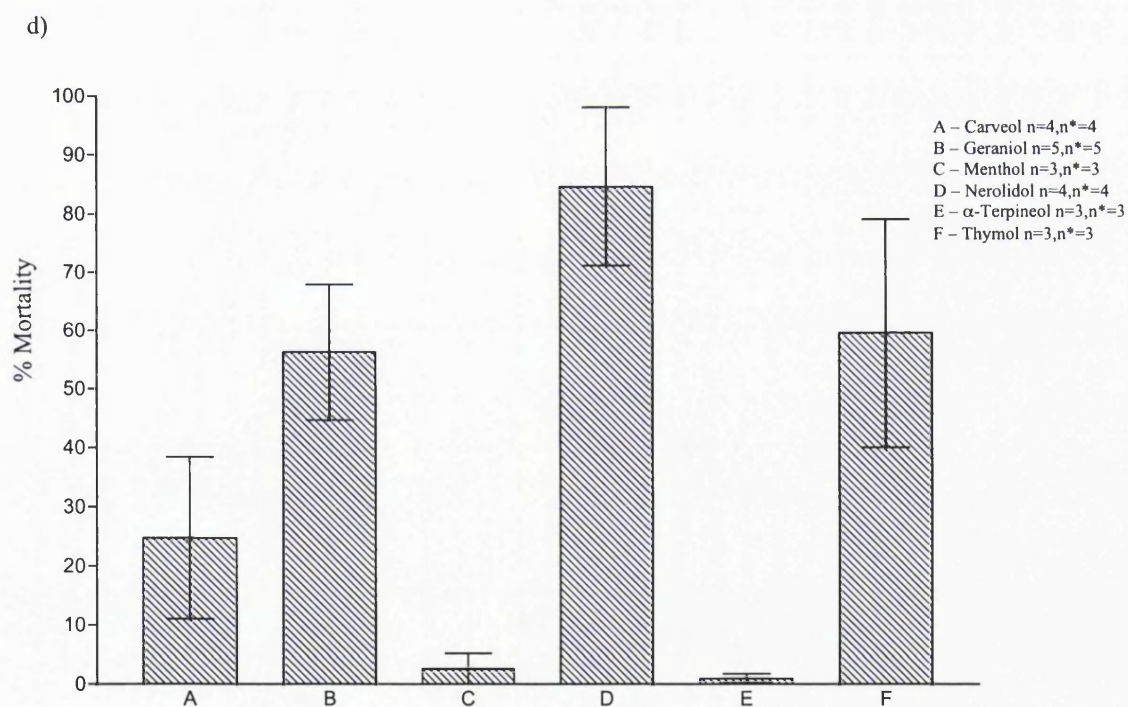
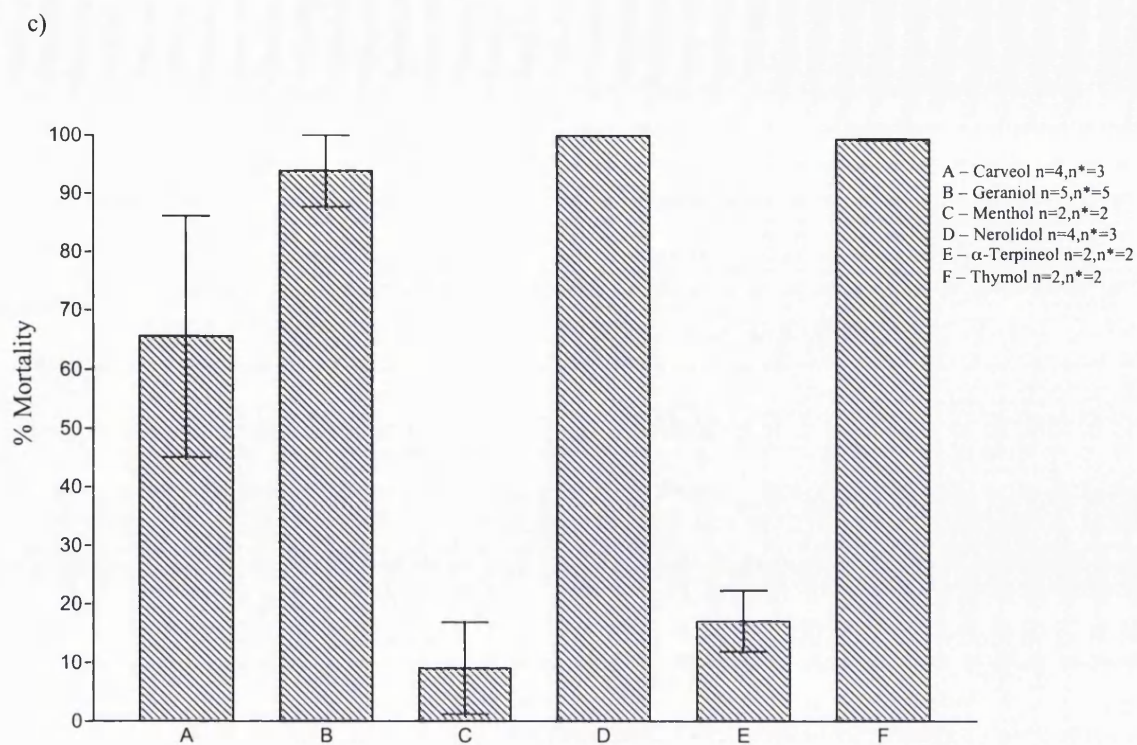


By analogy with the discussion of pediculicidal activity testing, the next phase of the *in vitro* work on ovicidal monoterpenoids should examine the effects of different formulations and methods of application, for example, the hair would normally be washed within 1-2 days of the product being applied. Similarly, the effects of monoterpenoids with and without washing of eggs with mild shampoo and rinsing after application could be studied.



**Figure 2.3 Ovicidal activity of monoterpenoids towards eggs of the human louse, *Pediculus humanus*, Part I**

Eggs were immersed in (a) 10 % and (b) 5 % solutions of the test agents for 10 min. The solvent was then evaporated and the eggs incubated. The percentage of eggs which did not hatch to give rise to viable nymphs (% mortality) was calculated as a percentage of the total number of eggs in the test. The % mortality data shown here have been adjusted for control mortality utilising Abbott's correction (see text). The number of replicates (n) for each test is shown alongside the number of these replicates where the control mortality was less than 20 % ( $n^*$ ), control mortalities for all other replicates were more than 50 %.



**Figure 2.3 Ovicidal Activity of monoterpenoids towards eggs of the human louse, *Pediculus humanus*, Part II**

Eggs were immersed in (c) 2 % and (d) 1 % solutions of the test agents for 10 min. The solvent was then evaporated and the eggs incubated. The percentage mortality was calculated and adjusted as described in Figure 2.3, Part I. The number of replicates (n) for each test is shown. The number of these replicates where the control mortality was less than 20 % is indicated (n\*), all other replicates had control mortalities of 20-22 %.

### ***Modes of action of essential oil constituents on louse eggs***

The data above compares the monoterpenoids on the basis of percent mortality, i.e. the total number of eggs rendered non-viable by the monoterpenoid. By observing the different stages at which each embryo was killed, MEC Ltd has studied the mode of action of compounds on louse eggs [52]: A lethal agent not dependent on a fully functional nervous system for ovicidal activity is able to kill eggs early on, at the 'undeveloped' stage (although observing lethality predominantly of the undeveloped nature does not preclude dual neuronal and non-neuronal activity). The presence of a correctly positioned black eyespot in the dead embryo is indicative of a stage of development that also features a fully functional nervous system; 'developed' eggs are therefore likely to have been arrested at this stage by specifically neuroactive compounds, as they were not able to kill the egg without the presence of a nervous system to mediate toxicity. Compounds resulting in 'half-hatched' eggs may have killed the nymphs via either a neuronal or a non-neuronal mechanism, but either way lethality did not occur until the nymph began to hatch; these compounds are generally lethal to lice and highly persistent, but are not able to penetrate the unperforated eggshell, and therefore act residually; when the louse breaks the operculum open upon hatching, residually-acting compounds are allowed to enter and thus kill the insect, approximately 2 weeks after their application to the egg. This activity can be confused with true ovicidal activity in clinical studies, due to the subsequent absence of live nymphs on the scalp. It should be noted that in the mortality studies of the previous section, eggs of the half-hatched type were included in the overall percent mortality. This would have had little impact on the outcome in this case as the number of half-hatched eggs resulting from monoterpenoid application was minimal (the mean percentage of half-hatched eggs resulting from monoterpenoid application was never more than 5 % above the mean control percent half-hatched). Future ovicidal studies should perhaps consider the merit of counting half-hatched eggs as 'hatched,' depending on the objectives of the test.

Although hatched, half-hatched, developed and undeveloped data were obtained in all ovicidal activity experiments, only a selection of this data is presented in Figure 2.4; the criteria for inclusion was the availability of at least two replicates per test compound with corresponding control mortality values of 22 % mortality or less. Because of the low number of replicates, the following discussion of the data should be treated as preliminary observations only.

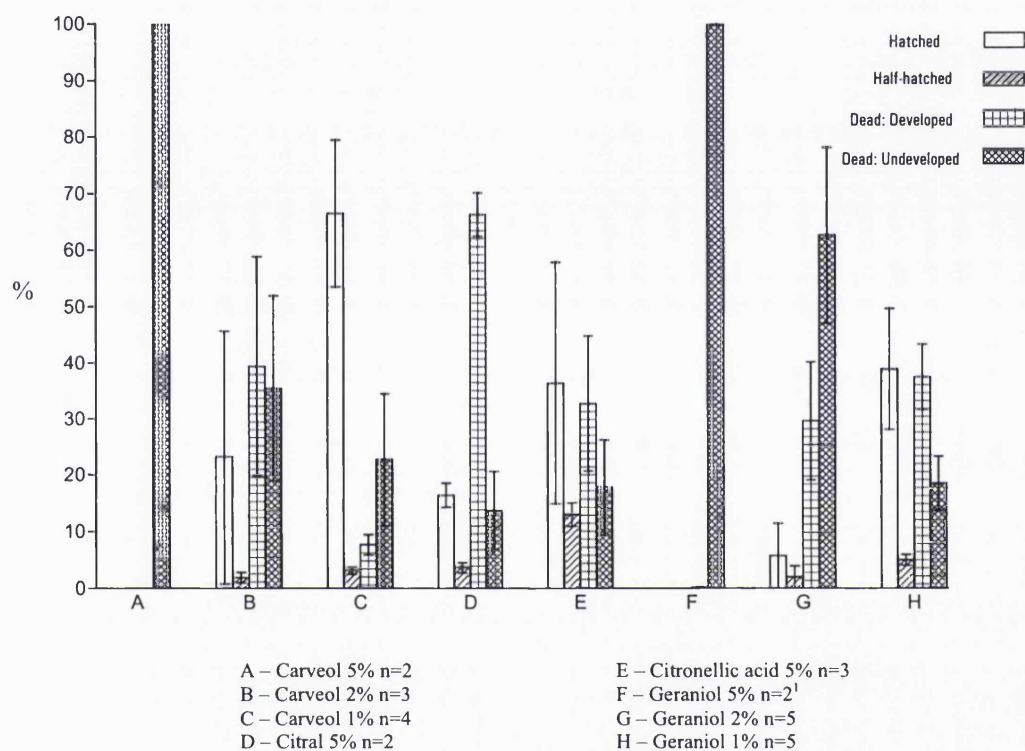
The data presented in Figure 2.4 suggest that monoterpenoids may not always kill eggs by the same mechanism; the stage at which the eggs are arrested in development by a particular compound may be concentration-dependent: carveol, geraniol and thymol at 5 % all leave eggs 98-100% 'undeveloped.' At 2 %, as the percentage of eggs in the undeveloped category decreases, instead of a concomitant increase in the 'hatched' category as might be expected, there is a large increase in the proportion in the 'dead' category; the increase in 'hatched' eggs is largely delayed until the concentration is decreased to 1 %. The effects of nerolidol appear to differ in that at 5% the predominant category is 'dead' and the proportion of undeveloped eggs is increased at 2 %. In fact, Figure 2.4 suggests that both neuronally-dependent and non-neuronally dependent mechanisms of death can occur as a result of application of many monoterpenoids, at particular concentrations. Furthermore, the relative frequency of mortality types in the ethanol-treated control group was the same as that of the control, again providing evidence that incubation in 100 % ethanol for short periods has no effect on louse eggs.

The data in Figure 2.4 suggest that most monoterpenoids do not generally give rise to 'half-hatched' lice, indicating a lack of residual activity of these essential oil constituents. Only two, citronellic acid 5 % and thymol 1 % raise the percent half-hatched above the minimal 0-6 % normally seen, giving 13 % and 7 % respectively. Because of their volatile nature, it is likely that most monoterpenoids will not persist long enough at sufficient concentration to have a residual effect.

Although currently more data is required to confirm these findings statistically, the trends illustrated in Figure 2.4 may have important consequences in terms of the use of monoterpenoids as ovicidal agents. For example, the data obtained for thymol suggest that lowering the concentration from 5 % to 2 % causes an 25 % decrease in the undeveloped category, presumably as the more resilient eggs resist the non-neuronally dependent action; instead of these eggs escaping to hatch, all except 2 % are then killed at the 'developed' stage, probably by a neuronally-dependent mechanism of action. If thymol was applied as an ovicidal agent such that initially all eggs were left at the undeveloped stage, the presence of another mode of action may prevent the compound losing efficacy in certain types of resistance, such as decreased target site sensitivity. However, this 'back-up' mode of action would probably not be beneficial in types of resistance such as those involving reduced entry of the active. The lack of residual action, even when the eggs and substrate are not washed after application, would also contribute to resistance avoidance.

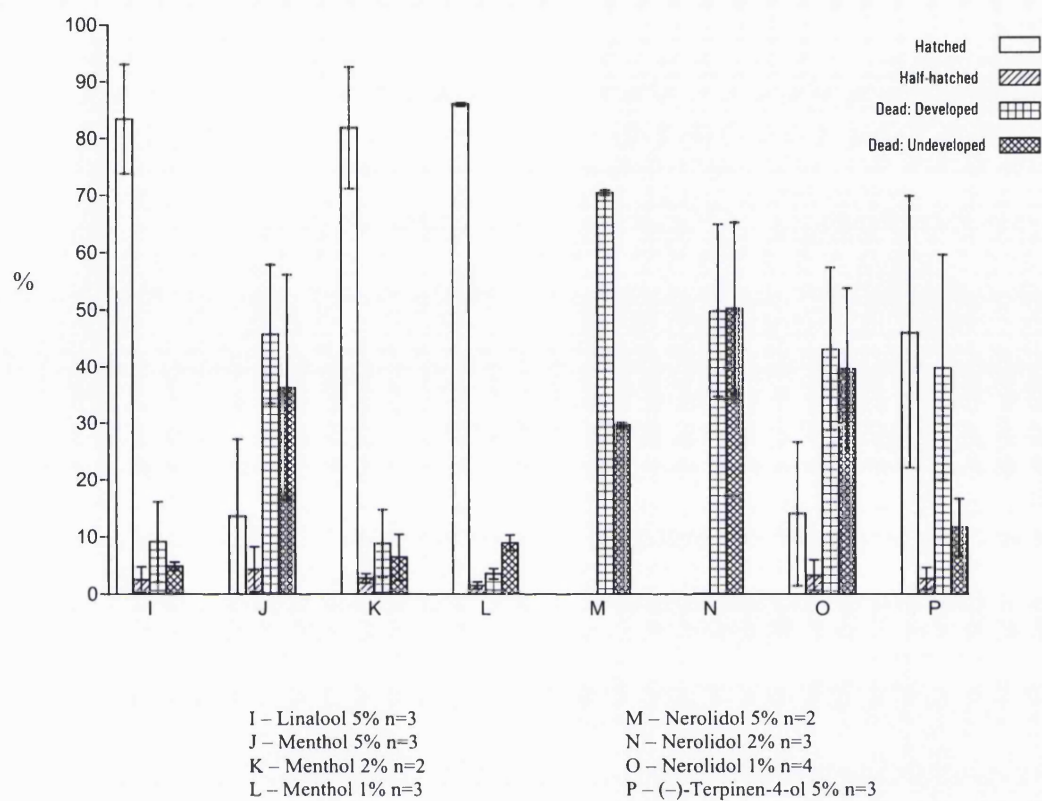


Even if the data obtained are more complex than perhaps anticipated, it has revealed that the ovicidal activities of at least several of the test substances may be highly dependent on an activity at neurones in the developing embryo, especially those of citral and nerolidol (see Figure 2.4). Although this lends weight to the theory that monoterpenoids can affect neurotransmission in a lethal manner, it appears unlikely that a single target can account for the observed dual effect.



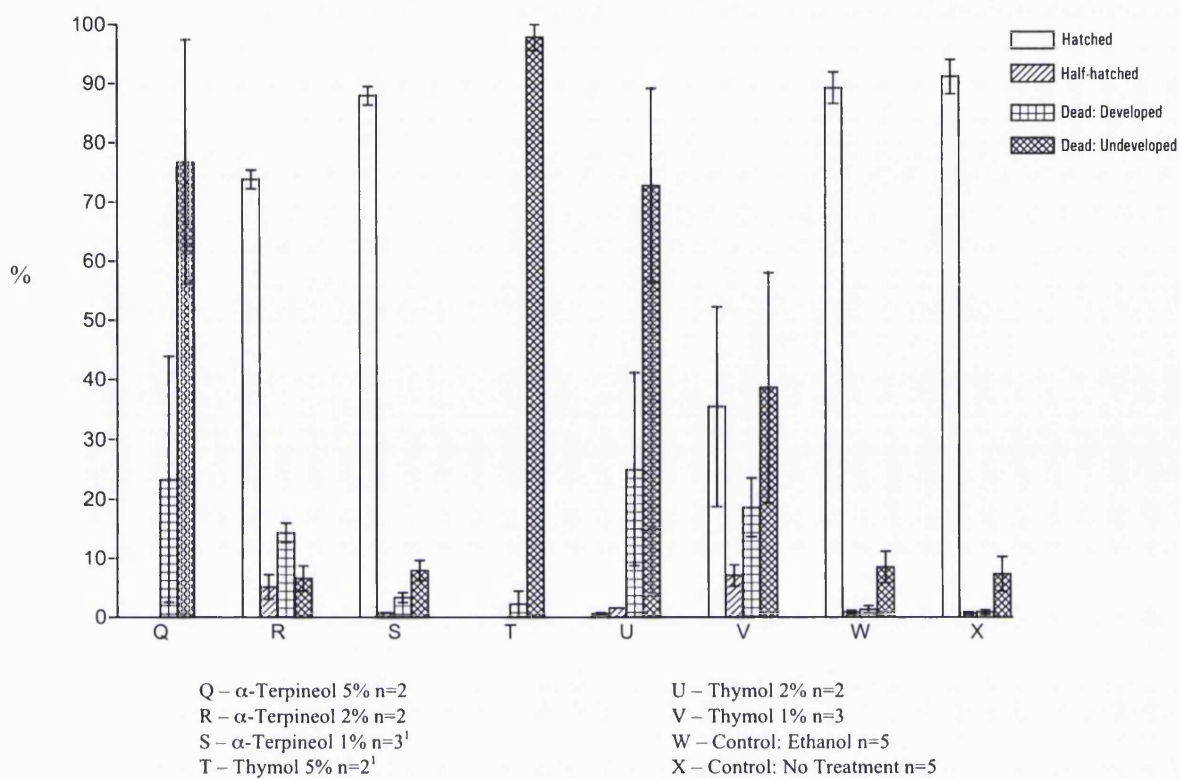
**Figure 2.4 (a) Mechanisms of action of ovicidal monoterpenoids on *Pediculus humanus* eggs**

This data is continued in Figure 2.4 (b) and (c). Eggs were dipped in 10 % solutions of test agents for 10 min and then incubated following solvent evaporation. When the control lice had hatched, the stage at which mortality occurred was assessed in all eggs. The test agent results have not been adjusted for corresponding control mortalities and, instead, typical control results are included for comparison in Figure 2.4 (c). Corresponding control mortalities were all 20 % or less for this data, except for those marked <sup>1</sup> where one replicate had a control mortality of 22%.



**Figure 2.4 (b) Mechanisms of action of ovicidal monoterpenoids on *Pediculus humanus* eggs**

Refer to Figure 2.4 (a) for details



**Figure 2.4 (c) Mechanisms of action of ovicidal monoterpenoids on *Pediculus humanus* eggs**

Refer to figure 2.4 (a) for details



### ***Improvements to the strategy for assessing ovicidal mechanism of action***

This assay, like the pediculicidal observational assay described previously in this Chapter, is another example of how a primary screen for lethality can be extended to gain additional information about the mode of action of a compound with carefully planned extensions to the protocol.

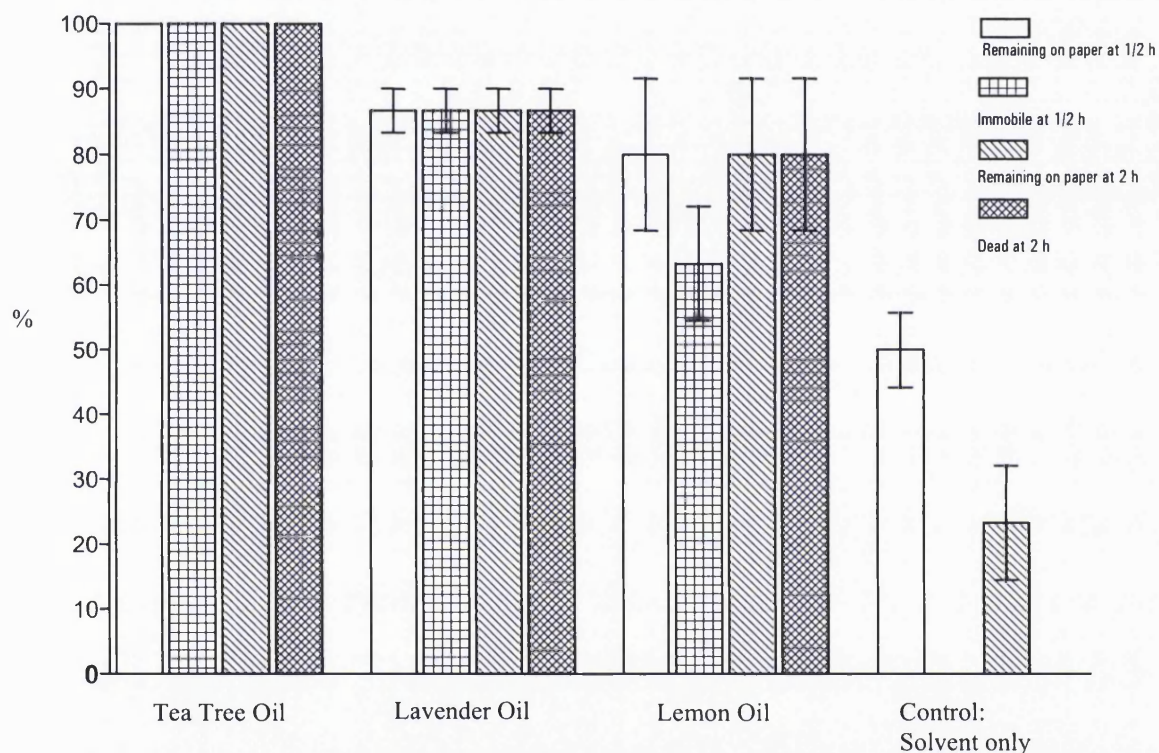
Whereas the observational data gained on lice described the manner in which different monoterpenoids appeared to have different modes of action, the results obtained in this study so far suggest that one monoterpenoid can have multiple lethal mechanisms of action on the same organism, the predominant one observed depending on the concentration. If monoterpenoids have multiple targets in lice it is perhaps not surprising that the behaviour of lice in the pediculicidal observational study was not easy to describe; the tremors and convulsions characteristic of neuroactive agents may have been masked by the symptoms arising from other biological effects.

This observation underlies the importance of standardising the doses applied in both pediculicidal and ovicidal mechanistic studies, and testing multiple concentrations. When ovicidal activity dose-response curves have been carried out for active terpenoids, mechanistic studies could then be carried out using a few, equipotent concentrations, for example, LD<sub>30</sub>, LD<sub>50</sub>, LD<sub>70</sub> & LD<sub>95</sub>. This would facilitate structure activity comparisons as, by removing the influence of potency from the mechanistic studies, compounds with a similar mode of action (but different potencies) would hopefully be easier to distinguish, thus facilitating the definition of multiple structure activity series.

#### ***2.2.4 The action of essential oils on house dust mites***

The most effective acaricidal essential oil in these tests was tea tree oil, as shown in Figure 2.5; it gave 100 % (0) immobility at 30 min and 100 % (0) mortality at 2h. Lavender oil was second most effective, taking into account both immobilisation and mortality data; it caused 87 % ( $\pm 3$ ) immobility at 30 min and 87 % ( $\pm 3$ ) mortality at 2 h. Lemon oil was least acaricidal, with 63 % ( $\pm 9$ ) immobility at 30 min and 80 % ( $\pm 12$ ) mortality at 2 h. None of the control group were affected. All of the results above are the mean of three observations with  $\pm$  one s.e.m shown in brackets. Only the mites remaining on the paper were scored, as there were some, particularly in the control group, which escaped from the

filter paper thereby terminating their contact with the test agent. The mites available for counting at the 30 min and 2 h time-points were generally all immobilised or dead, respectively, except in the control group (see Figure 2.5)



**Figure 2.5 The effects of essential oils on the house dust mite, *Dermatophagoides pteronyssinus***

Essential oils were used at 10 % v/v to impregnate filter papers, and the solvent (absolute ethanol) evaporated. Mites were placed on the papers and their escape routes limited. Mobility and mortality were assessed over time. Results are shown as the mean of 3 observations, with the s.e.m as error bars.

### ***The house dust mite as a model organism for pediculicide bioassay***

The results obtained in the pilot study to determine the effects of essential oils on house dust mites are suggestive of a correlation between insecticidal activity on lice and mites, as the rank order of activity for the three essential oils studied on *D. pteronyssinus* (tea tree, lavender and lemon oil) was the same as that found on lice. For this reason, and the fact that they reproduced highly successfully *in vitro* with the use of minimal resources, *D. pteronyssinus* showed potential as suitable model with which to screen for pediculicidal agents. The disadvantages of using them are their minute size, their incredibly high mobility and their translucency: The 'mite-chamber' was developed in this project with the aim of finding the most suitable apparatus with which to study small numbers of these acari, however, the problems of mites escaping from the test paper (which occurs via the thin wires holding the paper up) or being thrown off by movement of the apparatus could not be overcome. Handling the mites was also challenging; loading a test paper with 10 acari could take up to 20 min.

Perhaps the methods used in this thesis could be applied in other studies of *D. pteronyssinus*: the culture system developed by MEC Ltd is superior to many alternatives [see for example 131] in that it utilises substrates which are both hygienic and readily available. Furthermore, the mite-chambers developed here may prove useful in studying the behaviour of isolated mites; their dimensions allow them to be placed on to the stage of a light microscope, and can be inverted to follow the mites as they move around. The filter paper provides enough traction for them to cling to, and the bright white colour enhances the visibility of the creamy-brown mites. Because the filter paper with mites on is fixed inside a tube, the observer's hands are free to focus the microscope or move the mites with a brush.

The results obtained in this study indicate that the presence of mites on the test paper after a certain length of time is a good indicator of more acaricidal compounds, the test could therefore be used as a quick preliminary screen for activity, for example, in the bioassay-guided fractionation of plant extracts such as essential oils. If escape of mites via the supporting wires could be prevented this would enhance the accuracy of the test.

Acaricides have been shown to reduce allergen levels and clinical symptoms of house dust mite allergy when teamed with household cleansing regimes but, although both allergy to the house dust mite and mortality due to asthma are rising [83,154], none of the presently available acaricidal formulations are widely used, probably for reasons of cost, low efficacy, and consumer fears over potential adverse effects on humans, pets or furnishings. Some

natural products have previously been investigated for activity towards house dust mites, and tea tree oil was reported to be effective when added to laundry [131]. The utility of essential oils and their constituents as anti-dust mite agents around the home is debateable, as their volatile nature may aggravate the respiratory conditions they would be designed to help alleviate. Further investigation is clearly required.

Even though *D. pteronyssinus* are particularly easy to culture, they are very difficult to handle and observe. Although these preliminary tests suggest there may be a correlation between pediculicidal activity and acaricidal activity, if an alternative to the Orlando strain louse is required it may be best to consider another organism, perhaps a larger mite species, before working with *D. pteronyssinus*.

### **Summary and direction**

Thus far, the activities of a range of monoterpenoid constituents of essential oils have been confirmed on *P. humanus* adults. Some of these compounds also appear to be highly active against louse eggs. The evidence so far suggests that the rank order of volatile terpenoid activity on *P. humanus* ova may differ from that on adult insects, however, at this stage, the possibility that using a different application method in the ovicidal activity test influenced the order of potency cannot be ruled out.

Observation of lice and eggs during experimentation revealed that, upon exposure to certain monoterpenoids, physiological symptoms result which are reminiscent of those induced by neurotoxic pesticides. Past literature has identified mammalian GABA receptors as targets for several monoterpenoids; Chapter 3 of this thesis will investigate if pediculicidal and ovicidal terpenoids can influence the activity of insect GABA receptors, using a well established *in vitro* model.

## **Chapter 3**

### **Investigating the effects of monoterpenoids on GABA-mediated neurotransmission in insects**

#### **3.1 The use of a model insect GABA receptor as a starting point for mechanistic study**

Although there are some insect-lethal compounds with non-neuronal sites of action [79], the majority of current commercially successful insecticides act on targets in the nervous system, including acetylcholinesterase (AChE), nicotinic acetylcholine receptors (nAChR), Na<sup>+</sup> channels and GABARs [29,182].

The results presented in Chapter 2 of this thesis indicate that at least some monoterpenoids may act on neuronal targets in lice: The most effective pediculicidal monoterpenoids caused rapid incapacitation of adult insects. Behavioural studies also suggested that monoterpenoids could induce variable degrees of ataxia, malco-ordination, sudden loss of locomotion, or twitching; furthermore preliminary evidence suggests that some monoterpenoids at certain concentrations may cause substantial mortality in embryos in the later stages of development, when functional nervous systems are present, and not before.

Methods for studying a putative mechanism of action might include observing the distribution of terpenoid binding sites in insect tissue with a radio-labelled ligand, or terpenoid binding to isolated potential targets such as enzymes or receptors.

Monoterpenoids are generally lipophilic and therefore high levels of non-specific binding to cell membranes may hinder labelling studies. For this reason, pharmacological investigation of their activity at isolated insect tissues, enzymes or receptors may prove more fruitful than binding studies. As many insecticides affect the activity of ligand-gated ion channels, a member of this proteinaceous receptor superfamily would provide an ideal candidate for a putative monoterpenoid pharmacological target.

The study of compounds active at ligand-gated ion channels, like picrotoxinin (see Chapter 1), has been greatly facilitated by electrophysiology. Electrophysiological techniques can be

used to explore the fine details of neuronal ionotropic receptor pharmacology either *in situ* or when cloned and heterologously expressed; the activity of ion channel populations in cells and tissues can be monitored, or just the behaviour of single channels.

The insect GABA receptor model chosen by the Sattelle Laboratory to study insect GABAR-picrotoxinin interactions was employed in this investigation. This model, the RDL<sub>ac</sub> homomeric insect GABAR, has been proven to be suitable for the study of insect GABARs, as it faithfully reproduces several aspects of the pharmacology of the most frequently encountered native insect ionotropic GABARs: In Chapter 1 of this thesis, the pharmacologies of GABA<sub>A</sub> receptors, native insect GABARs and RDL homomers were compared; from this account it is clear that RDL homomers possess similar properties to the majority of insect receptors in terms of the agonist, antagonist and modulator profiles. The pharmacology of RDL homomers and most native insect receptors differs principally with respect to benzodiazepine sensitivity, which is interesting considering benzodiazepines appears to require both  $\alpha$  and  $\gamma$  subunits for potency [237], whereas other modulators, such as pentobarbital, can potentiate certain homo-oligomeric GABA<sub>A</sub> receptors [26]. This suggests that most native insect receptors may be hetero-oligomers, possibly consisting of a least one RDL-like polypeptide and one other subunit type.

Whether or not there are any native insect receptors that are homo-oligomers of RDL-like subunits is still not known: Single channel properties of heterologously expressed RDL homomers are reported to differ from those of GABARs on cultured, RDL-expressing *Drosophila* neurones [280,281], although the expression systems for these experiments were different, and this may have led to variations in post-translational modification or surrounding membrane chemistry [119].

The widespread occurrence of RDL-like subunits throughout insect nervous systems [12,103,221] and the fact that the A302S mutation has such radical consequences in terms of insecticide resistance in a number of species (described in Chapter 1), are other factors leading to the conclusion that RDL-like subunits are key determinants of insect GABAergic neurotransmission and pesticide action. The fact that RDL-like subunits exist in numerous insect species provides a rationale for investigating pediculicidal monoterpenoid activity on this *Drosophila* GABA receptor subunit; even though monoterpenoids can have different effects on different insects or insect stages, this is not necessarily due to interspecific differences at the target site level.

### **3.2 The use of the *Xenopus laevis* oocyte as an expression system for studying recombinant GABA receptors**

Oocytes of *Xenopus laevis*, a large South African clawed toad, were first used in the 1970s for the expression of microinjected foreign polynucleotides. Since the first experiments by Barnard, Sumikawa and Miledi [19,171,244], the *Xenopus* oocyte has now become an established *in vivo* expression system for mRNA and cDNA encoding neurotransmitter receptors and ion channels. The features of these cells which make them such useful tools for this purpose include their enormous reserves of the components required for protein synthesis, and their large size and distinct polarity which enable reliable microinjection of nucleotides into either the nucleus or the cytoplasm and facilitate 2-electrode voltage-clamp electrophysiological studies. From injected polynucleotides, *Xenopus* oocytes synthesise neuronal receptor subunits, assemble the polypeptides and insert the resulting multi-subunit receptor into the plasma membrane. Receptors expressed heterologously in *Xenopus* oocytes display the pharmacology and gating characteristics of those found in native tissue in the vast majority of cases; two isolated studies identified differences between the channel properties of certain nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus* oocytes and other cells [150,233], but no similar cases have been found for GABA receptors. The oocyte plasma membrane makes an ideal selective barrier such that the passage of ions through ligand-gated channels can be monitored by most electrophysiological methods. GABA receptor physiology/pharmacology was first studied by heterologous expression and electrophysiology in *Xenopus* oocytes by Miledi in 1982 [172].

### **3.3 Methods for RNA synthesis and *In vitro* expression of insect GABA receptors**

#### **3.3.1 Molecular biology**

##### ***Insect GABA receptor (RDL<sub>ac</sub>) subunit source, sub-cloning and vector isolation***

Wild-type Rdl<sub>ac</sub> cDNA was a gift from Dr. Richard Roush (Cornell University, U.S.A), it had been inserted into the vector pNB40 [36] (See Figure 3.1). Dr. Emmanuel Culetto of the Sattelle laboratory at The MRC Functional Genetics Unit, Department of Human Anatomy and Genetics, University of Oxford, U.K used this plasmid to transform competent *E. coli* DH5 $\alpha$  host cells. The plasmid was cloned and sub-cloned following established methods [118]. These procedures allowed replication of the vector and division of host cells to occur, in order to increase the plasmid copy number, thereby ensuring a high yield of RDL<sub>ac</sub> upon cell harvest. Dr. Culetto extracted the plasmid from *E. coli* using endotoxin-free maxi-prep kits (Qiagen, U.K.). Subsequent stages of RDL<sub>ac</sub> cDNA and cRNA synthesis were carried out by myself and are described later, they were adapted from the suggested protocols in the instruction booklet available for the Ambion “mMessage mMachine” SP6 RNA synthesis kit.

##### ***Quantification and qualitative analysis of extracted plasmid DNA***

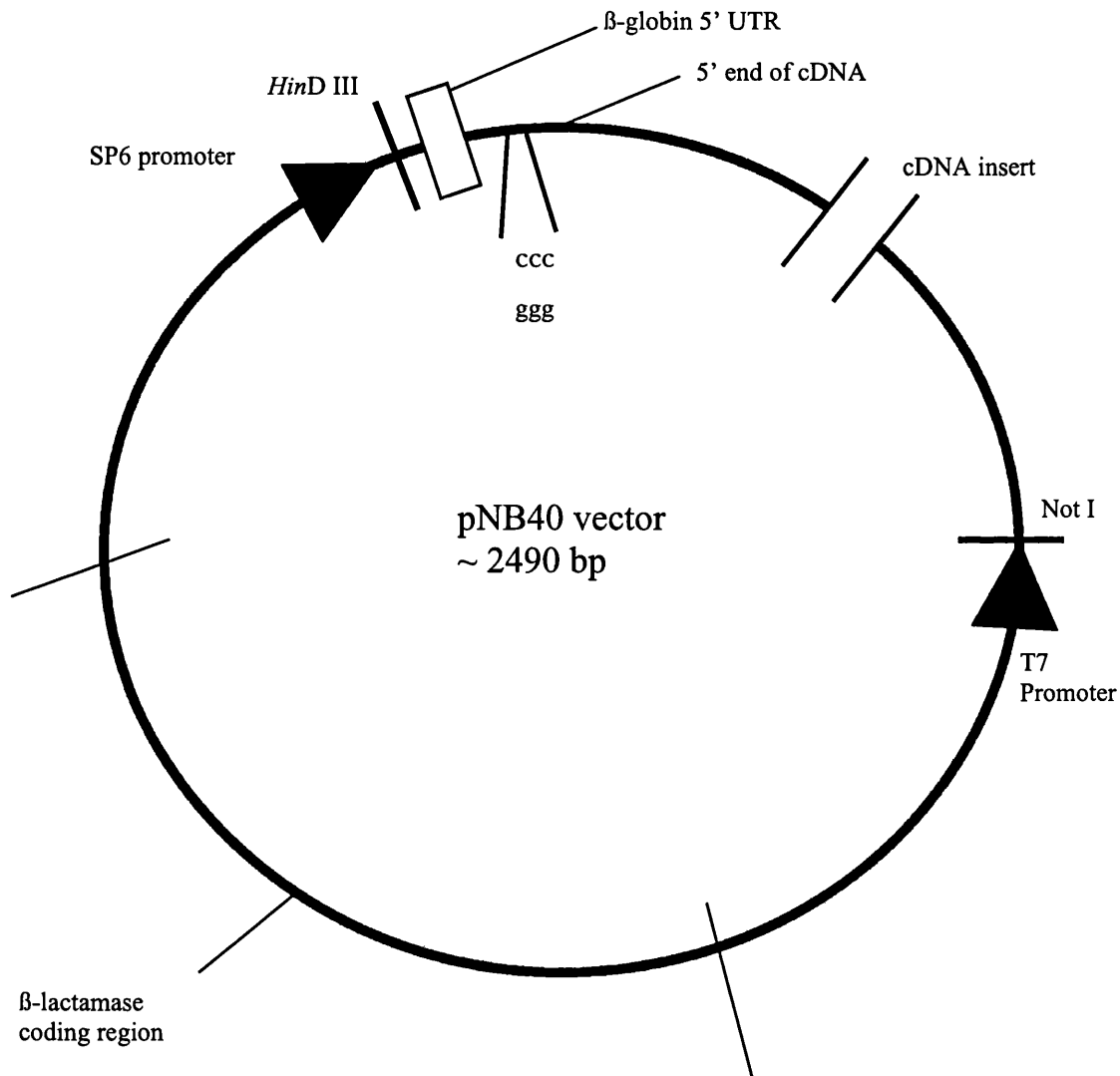
A sample of the plasmid DNA extract was run on an analytical ethidium bromide (EtBr) tris-acetate-EDTA (TAE)-agarose gel to examine its purity and integrity, and to estimate the DNA concentration. A 1 % TAE-agarose gel was prepared, to which EtBr was added as an intercalating stain at a final concentration of 0.2  $\mu$ g/ml, and left to set. When polymerisation was complete, TAE was poured over to a level of 0.5 cm above the surface, to act as a running buffer.

A mixture of plasmid DNA, 2  $\mu$ l, ultra-pure water ( $_w$ H<sub>2</sub>O), 14  $\mu$ l, and 10 x Orange G loading buffer, 4 $\mu$ l, was loaded onto the gel alongside a sample of 1 kb marker DNA, for later comparison, to estimate the plasmid concentration. Current was applied to mobilise the DNA and the resulting bands were viewed under U.V. light, which causes bound EtBr to fluoresce. The presence of one discrete band in the extract, corresponding to the plasmid DNA, was confirmed.



The exact concentration of plasmid DNA in the extract was quantified spectrophotometrically by measuring the optical density (OD), or absorbance of light, of a diluted sample at a wavelength of 260 nm ( $A_{260}$ ). A single OD unit is given as  $\sim \log_{10}$  (intensity of transmitted light)/(intensity of incident light). An OD of 1 at  $A_{260}$  corresponds to 50  $\mu\text{g}$  of double-stranded DNA (dsDNA) per ml. To obtain an accurate value of the plasmid DNA concentration using this method, a minimum cuvette concentration of DNA of 1 ng/ $\mu\text{l}$  in  $\text{H}_2\text{O}$  is recommended [75]. The amount of sample to add to the cuvette can therefore be gauged from the concentration of plasmid DNA estimated by TAE-agarose gel.

The ratio  $A_{260}/A_{280}$  was also calculated. Pure samples of DNA have  $A_{260}/A_{280}$  ratios of 1.8 and, as proteins absorb light with a wavelength of 280 nm, values of less than 1.8 indicate that the sample is contaminated by protein or phenol and should not be used.



**Figure 3.1 Plasmid pNB40, the transcription vector**

RDL<sub>ac</sub> cDNA is cloned into the polylinker site. The first strand of each cDNA was tailed with 10-15 cytosines, such that the second strand would be synthesised with a string of guanines at the 5' end, and the subsequent cRNA would bear the poly A tail characteristic of eukaryotic primary transcripts. The SP6 and T7 promoters are start sites for the respective bacteriophage RNA polymerase enzymes. The 5' untranslated sequence between promoter and cDNA insert is from sequences upstream of the *Xenopus laevis* β-globin gene, and is intended to facilitate transcription of heterologous cDNA in *Xenopus* oocytes. Hind III and Not I restriction endonuclease cleavage sites are also marked; Use of Not I prior to transcription linearises the plasmid, disconnects the T7 promoter from the insert, and provides a stop site for SP6 to synthesise a discrete transcript of sense cRNA. The β-lactamase gene confers ampicillin resistance, allowing selection of transformed bacterial colonies during cDNA cloning. The remainder of the sequences are derived from the bacterial plasmid Col E1.

### ***Preparation of linearised template DNA***

The extracted circular pNB40 plasmids were linearised, using the restriction endonuclease Not I, to provide an RNA polymerase stop site immediately after the  $RDL_{ac}$  gene. Cleaving the plasmid on the 3' end of the insert ensures that only sense mRNA strands (with the same nucleotide sequence as that found *in vivo*) will be synthesised when the SP6 mRNA polymerase is added. Not I also cuts dsDNA in a staggered fashion leaving a 5' overhang, this prevents non-specific transcription initiation, which can occur at 3' protruding ends.

The restriction digest comprised 10  $\mu$ l 10x NEB3 buffer, 1  $\mu$ l 100x bovine serum albumin (BSA), 10  $\mu$ g of DNA and  $H_2O$  up to 95  $\mu$ l, to which 5  $\mu$ l of 10 U  $\mu$ l<sup>-1</sup> Not I were added (1 U of enzyme will cut 1  $\mu$ g of DNA in one hour given optimal ionic conditions, which are provided for here by NEB3). The tube was flicked to mix the contents and droplets of liquid brought down by brief centrifugation. The reaction was incubated at 37 °C for 2h. Prior to termination of the reaction by SDS (sodium dodecyl sulphate) and proteinase K, (described in the following section), a sample of the restriction digest was run on a TAE-agarose gel (as before) against a sample of the original plasmid DNA to check the progress of the linearisation. A digest which has run to completion will migrate as a discrete band and to a shorter distance than the non-digested plasmid. The full protocol for this gel is described in a later subsection, "Confirmation of linearisation and purification by gel electrophoresis and quantification by spectrophotometry."

During the following procedures involving the synthesis and handling of RNA, all steps were taken to avoid contamination by RNases.

### ***Purification of the linearised template***

Before RNA synthesis can proceed, the DNA template must be purified to remove proteinaceous reagents used in previous stages, especially RNases. This is done by non-specific denaturation, precipitation and extraction of proteins by phenol/chloroform partition. These organic solvents precipitate proteins, leaving nucleic acids intact.

The protein denaturant, SDS was added to the restriction reaction to give a final concentration of 0.5 %. Proteinase K was added at a final concentration of 100-200  $\mu$ g/ml. This enzyme digests the denatured proteins leaving smaller polypeptide units, which require fewer phenol extractions to remove. The reaction was incubated for 1 h at 50 °C.

After this time, the reaction volume was increased by 150 % by the addition of  $\text{H}_2\text{O}$  to ensure a large aqueous phase upon partition.

To a final volume from above of 250  $\mu\text{l}$ , 200  $\mu\text{l}$  of phenol/chloroform/isoamylalcohol (IAA), 25:24:1, were added and the extraction vortexed for 5 s. Partition was completed by centrifugation at 12000 rpm for 5-10 min at 4 °C, as low temperature enhances phase separation. After centrifugation, three layers were apparent: a large upper aqueous phase, approx 300  $\mu\text{l}$ , containing the nucleic acids; a narrow interface, containing denatured proteins, and the lower phenol/chloroform phase. The aqueous phase was removed, avoiding contamination from the interface, and transferred to a new microfuge tube. The interface and lower phase were discarded.

Assuming the volume of the aqueous phase removed was 250  $\mu\text{l}$ , 50  $\mu\text{l}$  of  $\text{H}_2\text{O}$  and 200  $\mu\text{l}$  of chloroform were added. The tube was vortexed and centrifuged at 12000 rpm for 5-10 min at 4 °C. This step removes final traces of polypeptides and also phenol, which partitions into the chloroform layer. The nucleic acid-containing supernatant was removed, again avoiding the organic layer, and transferred to a new microfuge tube. To a volume of supernatant of 200-250  $\mu\text{l}$ , 3 volumes of absolute ethanol and 1/10 volume of sodium acetate (3 M, pH 5.2) were added. The tube was inverted gently to mix the contents and incubated at -20 °C for 1-18 h. Ethanol effectively precipitates polymeric nucleic acids at -20 °C or less in the presence of monovalent cations; precipitation allows concentration of the DNA by subsequent resuspension in a smaller volume.

The precipitated nucleic acids were pelleted by centrifugation at 13000 rpm for 30 min at 4 °C and the colourless pellet washed carefully by addition of 100  $\mu\text{l}$  of 70 % ethanol in RNase-free water. Washing the pellet removes salts, which inhibit transcription enzymes. The tube was centrifuged at 4 °C for 5-10 min at 13000 rpm to re-pellet the nucleic acids. All visible traces of ethanol were removed with a drawn-out, glass Pasteur pipette. The pellet was air-dried for 2-3 min by leaving the microfuge tube in an RNase-free laboratory environment with the cap off.

For transcription, the DNA template should be at approximately 0.5  $\mu\text{g}/\mu\text{l}$  such that the resulting RNA is not too dilute for injection into the oocytes. The pellet, which was assumed to consist entirely of linearised pNB40, was re-suspended in 15-20  $\mu\text{l}$  RNase-free water at room temperature for 15-20 min with the microfuge cap closed. The pNB40 concentration was estimated by agarose-gel electrophoresis (below) and adjusted if

necessary.

***Confirmation of DNA template linearisation and purification by gel electrophoresis and quantification by spectrophotometry***

The DNA template was examined for successful linearisation and purification by running the following samples on a TAE-agarose gel (prepared as before):

- 1 - Linearised and purified pNB40, 0.5µg; RNase-free water, 7µl; and a volume of 2x running buffer (Ambion, U.K.) equal to the total volume of DNA plus water.
- 2 - Non-linearised pNB40, 0.5µg; RNase-free water, 7µl; and a volume of 2x running buffer (Ambion, U.K.) equal to the total volume of DNA plus water.
- 3 - Marker DNA to enable the estimation of the linearised pNB40 concentration in sample i).

The exact concentration of the linearised template and proteinaceous contamination were evaluated by taking the  $A_{260}$  and  $A_{260}/A_{280}$  of a sample as described previously.

***In vitro transcription***

RNA was synthesised from the linearised plasmid template using an SP6 Message-Machine kit (Ambion, U.K.), reagents from which are denoted by '(kit)'. The following were added to an RNase-free microfuge tube: 2 µl Reaction Buffer, whirlimixed and centrifuged briefly before use (kit); 10 µl Ribonucleotide Mix, 2x, whirlimixed and centrifuged briefly before use (kit); 1 µg linearised plasmid, flicked and centrifuged before use; nuclease-free water (kit) to give a final volume of 18 µl; 2 µl 10x Enzyme Mix (kit), which contains DNA-dependent RNA-polymerase specific for the SP6 promoter and an RNase inhibitor, were added last. The tube contents were mixed by flicking and brief centrifugation, and incubated for 1.5 - 2 hr at 37 °C. The ribonucleotide mix contains the components of eukaryotic mRNA required for its assembly, these being all four nucleotides and a 7-methylguanosine cap analogue which is incorporated at the 5' end.

***Removal of the DNA template***

To yield a pure, quantifiable amount of RNA it was necessary to remove the DNA template, this was done by DNA-specific enzymatic digestion and precipitation: DNase I, 1 µl (kit) was added and the digestion incubated at 37 °C for 15 min. After this time, 30 µl nuclease-free water (kit) and 25 µl LiCl (kit) were added to precipitate the RNA, the tube

was flicked to mix the contents and then incubated at -20 °C.

After 0.5-1 h, the tube was centrifuged at 12 x 1000 *g* for 15 min at 4 °C to pellet the RNA. The supernatant was removed immediately and the pellet washed by addition of 500 µl 70 % ethanol in RNase-free water. The tube contents were thoroughly mixed prior to centrifugation at 12,000 x *g* for 5 - 15 min at 4 °C, to re-pellet the RNA. Using a drawn-out glass Pasteur pipette, all visible traces of ethanol were removed. The microfuge tube was left with the cap off in RNase-free laboratory conditions at room temperature for 5 min to evaporate the final traces of liquid. Between 15 and 20 µl nuclease-free water were added, depending on the pellet size, the tube capped and the RNA left at room temperature for 10 min to re-suspend.

#### ***Confirmation of synthesis and isolation of RNA, its aliquotting and storage***

A tris-borate-EDTA (TBE) analytical gel was run to confirm the integrity, homogeneity and purity of the prepared RNA. The RNA solution should contain no significant amounts of other nucleotides. All equipment used was either autoclaved, treated with H<sub>2</sub>O<sub>2</sub> solution or specialist RNase removing product, or purchased RNase-free. All solutions were either autoclaved or double nitrocellulose-filtered to remove deleterious proteins, or purchased RNase-free. A 1 % TBE - agarose gel was prepared with EtBr at 0.2 µg/ml. After the gel had partially cooled, it was poured into a gel tank to set. TBE was poured on top as a running buffer to a level of 0.5 cm above the surface. In a microfuge tube, 1 µl of RNA suspension was added to 5 µl blue loading buffer (kit) and 4 µl nuclease-free H<sub>2</sub>O (kit). The tube was incubated at 65 °C for 10 min to denature RNA secondary structure. The RNA sample was run alongside a molecular weight marker and visualised under U.V. as before. A single band confirmed the purity of the RNA and the homogeneity of its structure.

The concentration of the RNA solution and the degree of contamination are assessed in the same manner as described previously for DNA, by measuring  $A_{260}$  and  $A_{260}/A_{280}$ . The RNA suspension was diluted in an RNase-free microfuge tube in RNase-free H<sub>2</sub>O. The approximation of the RNA concentration in the suspension from the gel enabled a sufficient amount of RNA to be included to give a reliable OD reading, as this requires at least 1 µg/ml [75]. The tube was flicked, quick-centrifuged and the contents transferred to a quartz cuvette.

The  $A_{260}$  was measured (relative to RNase-free water) and the concentration of RNA estimated by applying the formula:

$1 \text{ OD} = 1 \times A_{260} \text{ of ssRNA} = 40 \mu\text{g/ml}$ . The  $A_{280}$  was also taken, the degree of protein contamination of the RNA sample being given by the ratio  $A_{260}/A_{280}$ , which should be between 1.5 and 1.9.

The RNA sample was diluted with RNase-free water to give a final cRNA concentration of  $1 \mu\text{g}/\mu\text{l}$  and divided into aliquots of  $3 \mu\text{l}$ . Each aliquot provided sufficient injections of 50 ng for 60 eggs. The RNA was stored in a manner which minimised trauma to preserve its integrity. Batches of RNA about to be used or being subjected to multiple rounds of defrosting and refreezing were stored at  $-20^\circ\text{C}$ . RNA to be stored for long periods was frozen to  $-70^\circ\text{C}$ .

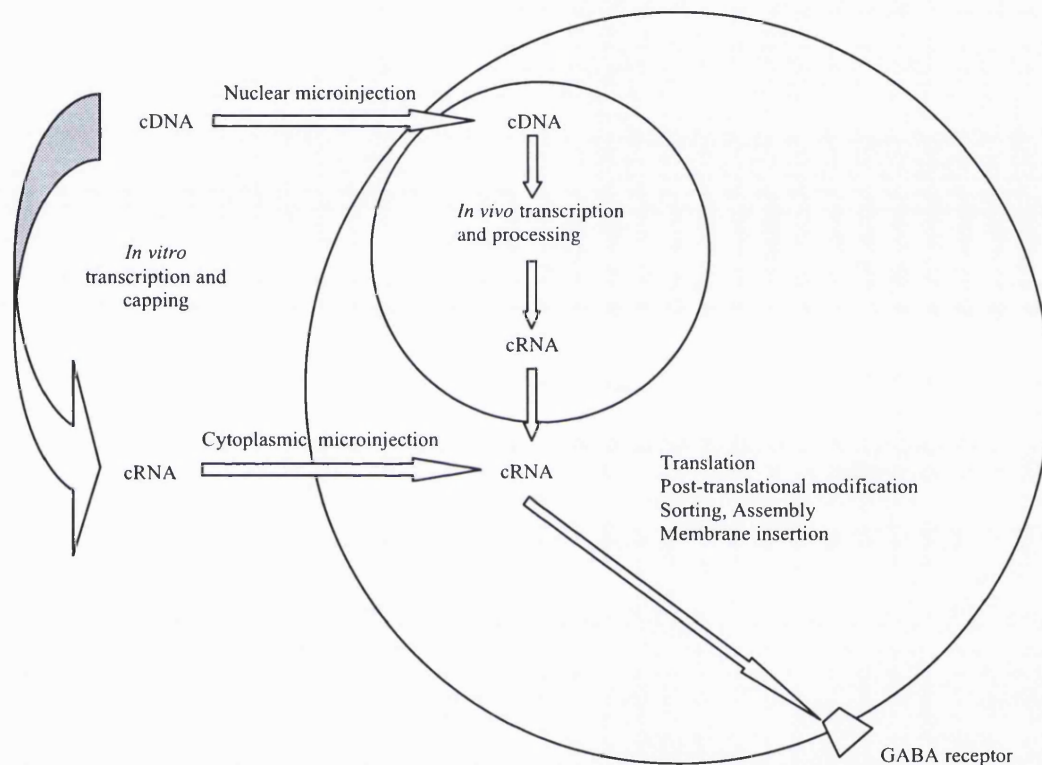
#### ***Xenopus laevis* oocyte preparation and cRNA injection**

Ovaries from mature *Xenopus laevis* females were the gift of Prof. D. Sattelle, when resident at The Babraham Institute, Cambridge, and Professor T. Smart, Department of Pharmacology, The School of Pharmacy, University of London. The ovaries were washed and stored for up to 24 h before use in Standard Oocyte Saline (SOS), which has the following composition (mM): NaCl, 100; KCl, 2;  $\text{CaCl}_2$ , 1.8;  $\text{MgCl}_2$ , 1; HEPES, 5. The pH was adjusted to 7.6 by addition of NaOH, 10 N.

Healthy oocytes at stages V and VI of development were selected for experimentation. An incubation of up to 2 min in collagenase type IA (Sigma, U.K.), 2 mg/ml in  $\text{Ca}^{2+}$ -free SOS, was used to ease removal of the follicle layers that envelope the oocyte. The  $\text{Ca}^{2+}$ -sensitive collagenase was diluted in a version of SOS with  $\text{CaCl}_2$  omitted and clumps of oocytes were washed thoroughly in  $\text{Ca}^{2+}$ -free SOS before and after collagenase treatment. Detachment of the follicle cells was completed manually using fine forceps. cRNA encoding  $\text{RDL}_{\text{ac}}$ ,  $1 \mu\text{g}/\mu\text{l}$ , was injected cytoplasmically using a Nanoject pipette (Drummond, U.K.). Each oocyte received 50 ng of RNA except for those left uninjected for use in control experiments (see Figure 3.2).

All oocytes were transferred to Petri dishes containing incubation medium. This consisted of SOS supplemented with antibiotics and an energy source as follows: penicillin 100 units/ml, streptomycin  $100 \mu\text{g}/\text{ml}$ , gentamycin  $50 \mu\text{g}/\text{ml}$ , sodium pyruvate 2.5 mM, and horse serum 10 ml/l. In the 30 min following injection, oocytes were kept at  $4^\circ\text{C}$  to allow

recovery. The cells were then incubated at 16 °C and transferred to fresh incubation medium on a daily basis. Electrophysiological recording began when expression was sufficient, usually 18 h after injection. Batches of cells showing consistent expression with large currents were moved to 4 °C to prolong their life. Such cells were viable for 4-8 days.



**Figure 3.2 *Xenopus* oocyte cDNA and cRNA microinjection**

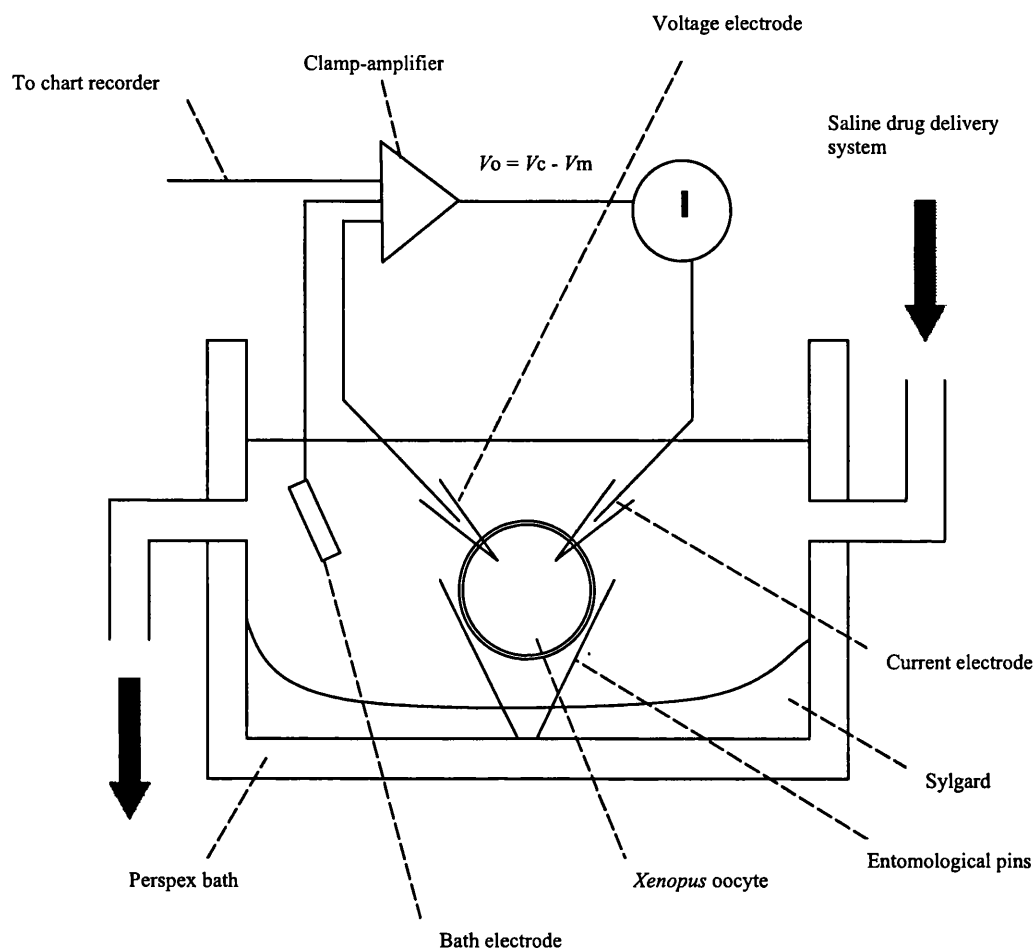
Many foreign proteins are modified and targeted appropriately in *Xenopus* oocytes. The genetic material enabling their synthesis is introduced by microinjection of either DNA or RNA into the nucleus or cytoplasm, respectively: Cytoplasmic injection was performed in the experiments described in Chapter 3, and nuclear injection in those in Chapter 4. Note that cRNA is a form of mRNA.



### 3.3.2 Electrophysiology

Individual oocytes were secured in an 80  $\mu$ l perspex bath by a surrounding ring of stainless steel entomological pins. The pins were embedded into a layer of the polymer Sylgard, which lined the chamber. The bath was perfused continuously with SOS by a gravity-fed system, at a flow rate of 5 ml/min, which allowed administration of SOS and test drugs. The oocyte membrane potential was held at -60 mV and membrane currents were monitored by 2-electrode voltage-clamp, using 3 M KCl-filled electrodes (0.5-5  $M\Omega$ ) and an Oocyte Clamp OC-725C amplifier (Warner Instrument Corporation, U.S.A). Signals were displayed on a chart recorder. A schematic representation of the bath and circuit is shown in Figure 3.3.

GABA was dissolved in SOS to give a 0.1 M stock solution, made fresh each day, and a serial dilution performed to obtain test concentrations ranging from 1 mM to 10 nM. Test compounds (monoterpenoids and eugenol) were dissolved in SOS to 5 mM with the aid of a small amount of acetone or DMSO (dimethylsulphoxide), and then diluted with SOS. The concentration of organic solvent in the test solutions did not exceed 0.1 %. Solutions of 0.1 % acetone or DMSO had no effect on the current required to clamp injected oocyte membranes at -60 mV, nor did it affect responses to GABA. DMSO and acetone have been used as solvents for drugs in oocyte electrophysiology on previous occasions [113].



**Figure 3.3** Diagram of the disposition of the *Xenopus* oocyte and electrodes in the perspex recording chamber used in voltage-clamp electrophysiology

The oocyte rests in a bath that is continually perfused with fresh saline. The voltage electrode, together with the bath or 'reference' electrode, monitors changes in the oocyte membrane potential resulting from intrinsic ion channel activity. The clamp-amplifier constantly compares the actual membrane potential ( $V_m$ ) with the desired, or command, clamp potential ( $V_c$ ) set by the researcher. The clamp-amplifier must increase  $V_m$  by a specific amount, the output voltage ( $V_o$ ), to maintain  $V_c$  at all times. This is achieved by the injection of current ( $I$ ) into the cell. The amount of current injected is always exactly equal, but of opposite polarity, to the net current flow through the channels. The current injected (in amps) is recorded and displayed by the clamp-amplifier. The change in amps injected over time can be displayed on a chart recorder as a trace.

GABA was applied in doses of 15-30 s duration, separated by a 3-10 min SOS wash to avoid receptor desensitisation. Oocytes expressing RDL<sub>ac</sub> were rejected if the responses to three identical GABA applications were not consistent. Terpenoids and eugenol were tested for activity on RDL<sub>ac</sub> GABARs by administering the compound alone at 100 µM for 2 min, which allowed equilibration with any binding sites, and then co-administering with 10 µM GABA for 15 - 30 s. GABA, 10 µM, was close to a previously published EC<sub>50</sub> for GABA [117], and therefore would elicit responses which were sensitive to either inhibitory or potentiating compounds. After each co-application, SOS was allowed to wash through for 10 min. This was followed by a further application of GABA to assess the reversibility of the terpenoid effect. Different terpenoids were tested on different oocytes, as the picrotoxane terpenoids were found to be incompletely reversible when tested in the same system [117]. Each terpenoid was screened on several RDL<sub>ac</sub>-expressing eggs (n = 3 or more from at least 2 batches). The effects of terpenoids on uninjected oocytes were also examined.

To further investigate the effects of GABA-potentiating compounds, a dose-response curve for GABA was generated from which the EC<sub>20</sub> was estimated. GABA was applied to oocytes in increasing concentration until the maximum response was obtained. Data for each oocyte were normalised with respect to the maximum response, dose-response data for individual oocytes were then pooled, averaged and presented ± one standard error of the mean (± s.e.m.) of experiments on *n* cells. GraphPad Prism (GraphPad Software, U.K.) was used to fit the four-parameter logistic equation below, which describes a sigmoid curve of variable slope, to the averaged, normalised data:

$$\varphi = I_{\min} + (I_{\max} - I_{\min}) / (1 + 10^{(\log EC_{50} - [A]) n_H})$$

where  $\varphi$  is the normalised current induced by a given concentration of agonist, [A];  $I_{\max}$  and  $I_{\min}$  are the maximal and minimal normalised agonist responses, respectively; EC<sub>50</sub> is the concentration of agonist predicted to elicit half the maximal response and  $n_H$  is the slope (Hill) coefficient.

Dose-response data for GABA-potentialiation by monoterpenoids and eugenol at recombinant RDL<sub>ac</sub> insect receptors were collected by applying a set concentration of terpenoid for 2 min, followed by co-application of this concentration with EC<sub>20</sub> GABA. This regime was repeated using increasing concentrations of test agent with a 10 min

washout between each. Data were handled in the same manner as for the GABA dose-response curve above, except that each response was normalised to the EC<sub>20</sub> GABA response for that cell. EC<sub>20</sub> GABA was applied regularly throughout to ensure reversal of the potentiating effect during washout.

#### **Source of materials**

Terpenoids and eugenol were obtained from Sigma (U.K.). GABA was obtained from RBI (U.K.).

### **3.4 Results**

#### **3.4.1 Thymol potentiates the action of GABA at recombinant homomeric *Drosophila melanogaster* RDL<sub>ac</sub> Insect GABA receptors**

The GABA dose-response curve obtained from oocytes expressing RDL<sub>ac</sub> is shown in Figure 3.4. From this an EC<sub>20</sub> value for GABA of 4  $\mu$ M was estimated for use in further experiments to investigate the potentiation by monoterpenoids. The EC<sub>50</sub> was 8.5  $\mu$ M (95 % confidence interval: 7.1-10.0  $\mu$ M), which was close to previously published EC<sub>50</sub> values for this receptor [24].

Thymol, 1  $\mu$ M - 100  $\mu$ M, potentiated the GABA response when co-applied for 15-30 s with EC<sub>20</sub> GABA, following a 2 min pre-application with thymol alone (Figure 3.5). The potentiation was fully reversible: applications of GABA after a 10 min washout resulted in currents identical to the control response (Figure 3.5). Potentiation was dose-dependent with respect to thymol concentration, for example, 10  $\mu$ M thymol potentiated the response to EC<sub>20</sub> GABA to a lesser degree ( $163 \pm 22$  %,  $n = 7$ ) than 100  $\mu$ M thymol ( $715 \pm 85$  %,  $n = 6$ ) (Figure 3.8). The degree of potentiation by thymol also appeared to be dependent upon GABA concentration: 100  $\mu$ M thymol increased the response evoked by 10  $\mu$ M GABA less than 2-fold, however the response to EC<sub>20</sub> GABA (4  $\mu$ M) was potentiated over 7-fold. Potentiation by concentrations of thymol greater than 100  $\mu$ M could not be investigated as they caused instability of the clamped current and usually resulted in death of the oocyte. Concentrations of thymol of 100  $\mu$ M were without effect in uninjected oocytes ( $n = 3$ , from separate batches).

### **3.4.2 Thymol has direct agonist activity at heterologously expressed *Drosophila melanogaster* RDL<sub>ac</sub> GABA receptors**

A direct effect of thymol was seen in RDL<sub>ac</sub>-injected oocytes: Concentrations of 1-50  $\mu$ M thymol and below had a negligible effect, eliciting changes in the clamp potential of 15 nA or less. Thymol, 100  $\mu$ M, resulted in currents which were  $57 \pm 22$  nA ( $n = 4$ ) in amplitude at 2 min (Figure 3.5), and therefore were very small in comparison to the corresponding potentiated EC<sub>20</sub> current, which was 47  $\mu$ A on average from the same cells. The responses to thymol alone began immediately upon application but were very slow to gain amplitude and failed to plateau throughout the 2 min application time. This current was not the result of a change in electrochemical gradient across the cell, as the pH of the bathing solution was not affected by thymol.

### **3.4.3 Eugenol and carvacrol have similar activities to thymol at Rdl<sub>ac</sub> homomers**

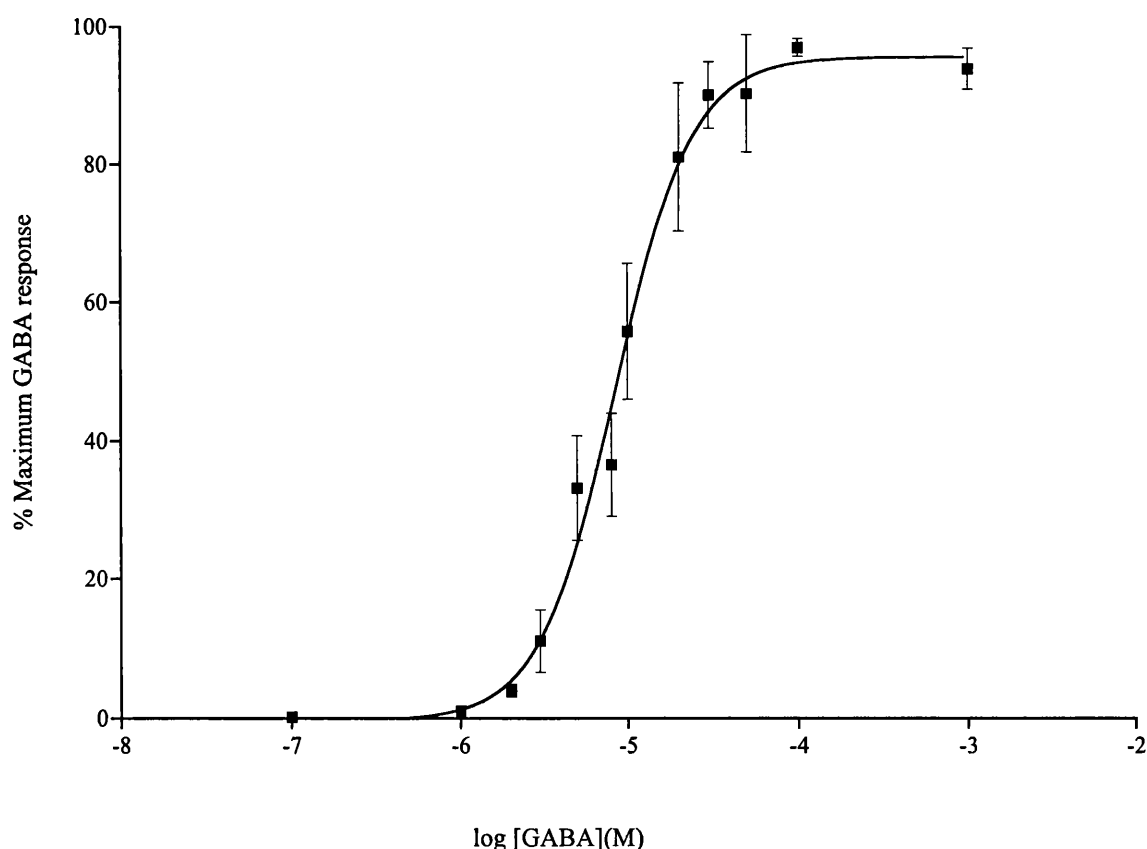
Co-application of eugenol, 10  $\mu$ M – 1 mM, with EC<sub>20</sub> GABA, after a 2 min pre-application with eugenol alone, also resulted in a dose-dependent potentiation of the GABA response (Figure 3.6). Eugenol, 100  $\mu$ M, increased the response to EC<sub>20</sub> GABA 2-fold ( $231 \pm 18$  %,  $n = 4$ ), whereas 1 mM eugenol gave a 17-fold potentiation ( $1713 \pm 52$  %,  $n = 3$ ) (Figure 3.8). Potentiation of GABA activity by eugenol was completely reversible: GABA applications after a 10 min washout generated responses that were identical to the controls (Figure 3.6).

In oocytes expressing RDL<sub>ac</sub>, eugenol, 10 $\mu$ M – 500  $\mu$ M, applied alone had a negligible effect; at 500  $\mu$ M, currents were 16 nA or less. Eugenol, 1 mM, alone elicited currents of which the average was  $42 \pm 19$  nA ( $n = 3$ ) in amplitude, this was very small compared to the average 1 mM eugenol-potentiated EC<sub>20</sub> GABA current in the same cells, which was 17  $\mu$ A. These responses were slow to gain amplitude and failed to plateau during the 2 min application time (Figure 3.6). The application of eugenol, 1mM, to uninjected oocytes evoked no detectable response ( $n = 3$ ).

Co-application of 0.1 – 100  $\mu$ M carvacrol with EC<sub>20</sub> GABA, after a 2 min pre-application with carvacrol, also potentiated the EC<sub>20</sub> GABA response (Figures 3.7 and 3.8). At 100  $\mu$ M

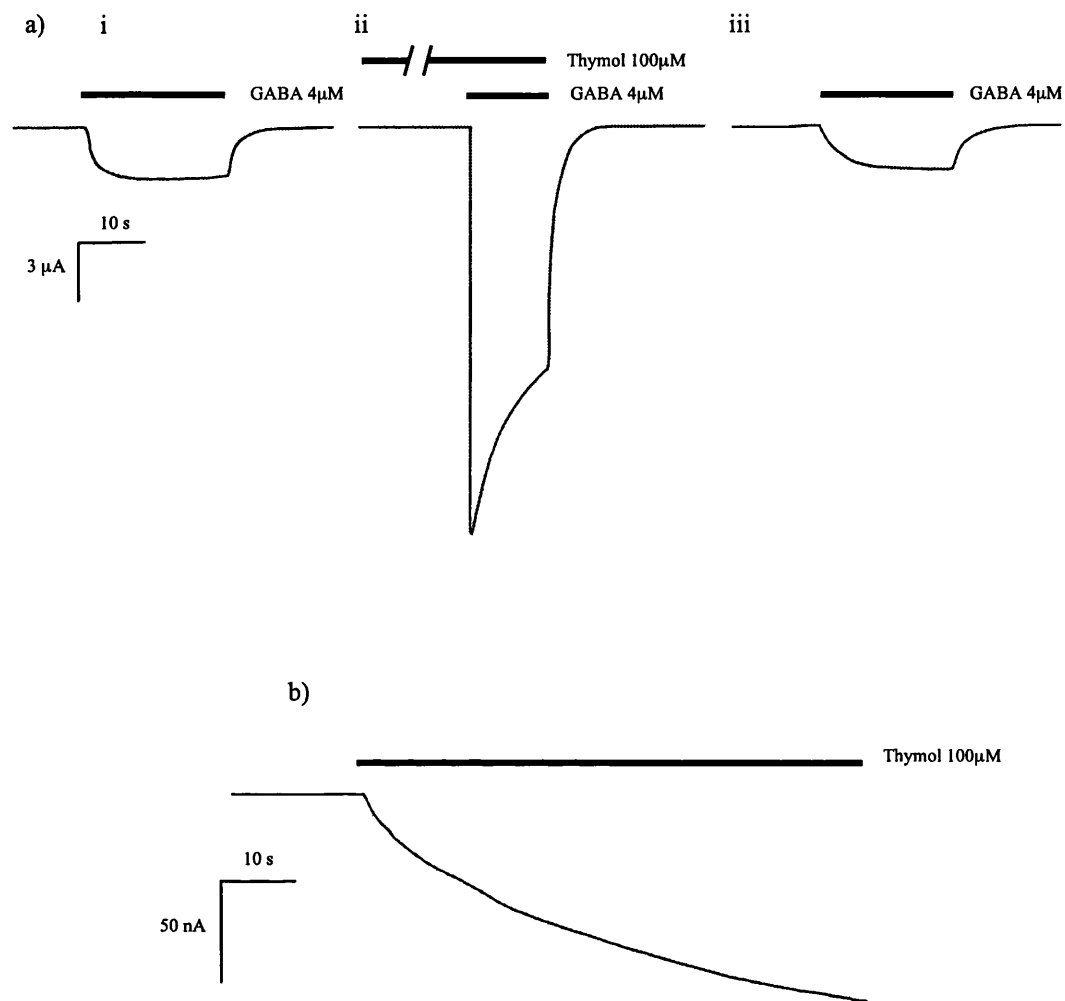
the response was  $362 \pm 49$  % of control ( $n = 6$ ). The GABA-potentiating activity of carvacrol was also completely reversible after a 10 min washout (Figure 3.7).

Applied alone, carvacrol  $0.1 \mu\text{M} - 50 \mu\text{M}$  had only negligible effects on the clamp current in RDL<sub>ac</sub>-expressing oocytes,  $50 \mu\text{M}$  carvacrol induced currents of less than 10 nA. At  $100 \mu\text{M}$ , the average current was  $26 \pm 11$  ( $n = 4$ ) nA in amplitude in comparison to the average carvacrol-potentiated EC<sub>20</sub> GABA current in the same cells, which was  $24 \mu\text{A}$ . The agonist responses of carvacrol showed similar kinetics to those of thymol and eugenol (Figure 3.7). The application of  $100 \mu\text{M}$  carvacrol to uninjected oocytes evoked no detectable response (currents  $< 3\text{nA}$ ,  $n=3$ ).



**Figure 3.4 The GABA dose-response curve obtained from *Xenopus* oocytes expressing the *Drosophila melanogaster* GABA receptor subunit RDL<sub>ac</sub>**

Oocyte membranes were voltage clamped at  $-60 \text{ mV}$ . Each point is shown  $\pm$  one s.e.m and is the mean of 4 – 11 observations. The EC<sub>50</sub> on this curve is  $8.5 \mu\text{M}$  (estimated by GraphPad Prism), similar to a previously published EC<sub>50</sub> for this receptor (see text). The EC<sub>20</sub> was estimated from the curve by eye at  $4 \mu\text{M}$ .



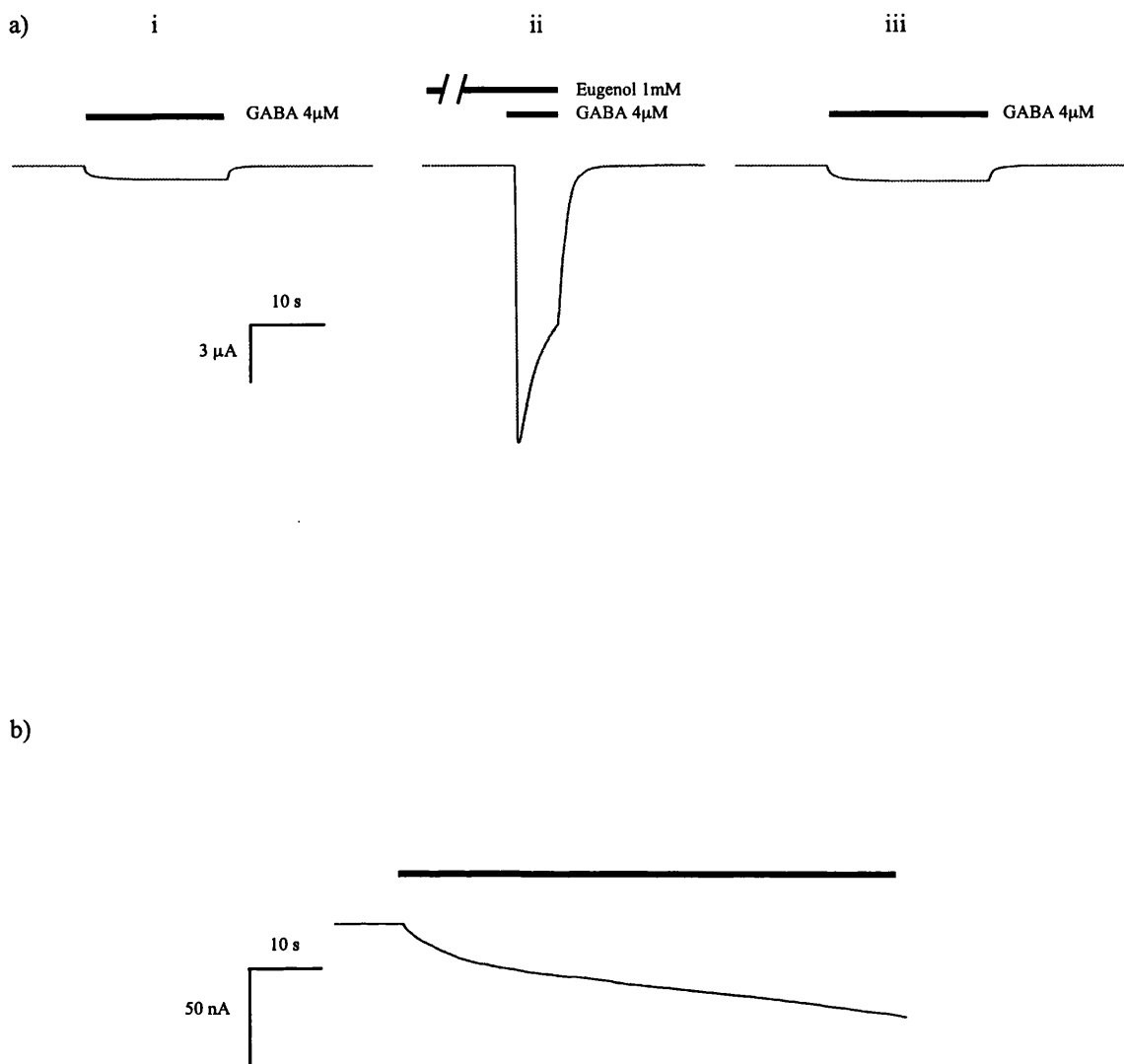
**Figure 3.5 Effects of thymol at an insect GABA receptor**

In *Xenopus* oocytes expressing the *Drosophila* GABA receptor subunit RDL<sub>ac</sub> voltage-clamped at -60 mV:

(a) thymol potentiates the EC<sub>20</sub> GABA response

- i. The response to EC<sub>20</sub> GABA applied alone.
- ii. EC<sub>20</sub> GABA co-applied with 100 μM thymol after a 2 min thymol pre-application.
- iii. The response to EC<sub>20</sub> GABA after a 10 min washout.

(b) thymol, 100 μM, applied alone shows agonist activity



**Figure 3.6 The effects of eugenol on an insect GABA receptor**

Responses to eugenol recorded from *Xenopus* oocytes expressing RDL<sub>ac</sub> homomeric GABA receptors, voltage-clamped at  $-60$  mV:

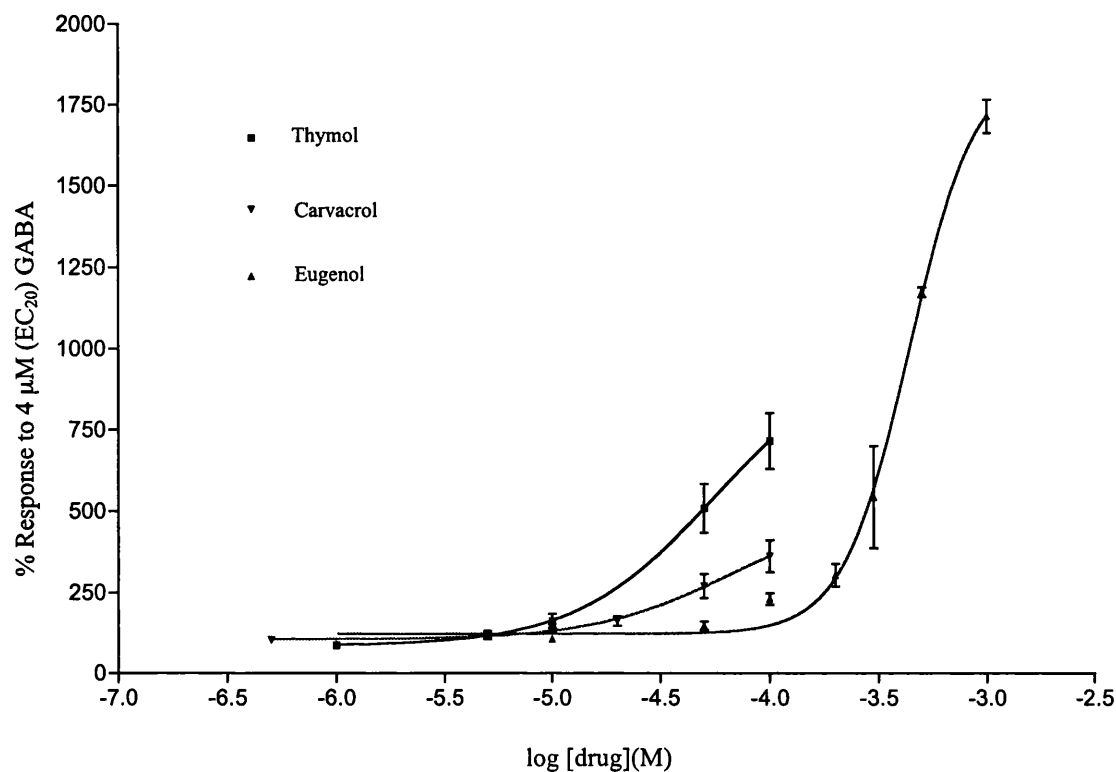
(a) when co-applied with EC<sub>20</sub> GABA

- i. Control response to EC<sub>20</sub> GABA.
- ii. The response to EC<sub>20</sub> GABA co-applied with 1 mM eugenol after a 2 min application of eugenol alone.
- iii. The control GABA response after a 10 min washout period.

(b) when applied alone at 1 mM







**Figure 3.8 Thymol, eugenol and carvacrol potentiate the GABA response of RDL<sub>ac</sub>- containing insect ionotropic GABA receptors**

The *Drosophila* GABA receptor subunit, RDL<sub>ac</sub> was expressed in *Xenopus* oocytes, and the oocyte membranes held at  $-60$  mV. Thymol, eugenol and carvacrol were co-applied with EC<sub>20</sub> GABA. Currents are expressed as a percentage of the control response to GABA, shown here as 100 %. Data points are displayed  $\pm$  one s.e.m. Each point is the mean of  $n$  observations,  $n = 6-7$  for thymol,  $n = 3-4$  for eugenol, and  $5-7$  for carvacrol.

### 3.5 Action of monoterpenoids on insect GABA receptors

#### 3.5.1 A novel activity for monoterpenoids

The effects of certain monoterpenoids and other volatile natural products on the ionic activities of nerve and muscle cells of vertebrates have been investigated previously [35,146,195]. There is also evidence of equivalent effects in some invertebrate tissues [70,138]. Certain monoterpenoids and eugenol have been shown to interact with heterologously expressed vertebrate neuronal receptor targets [176,285] but, thus far, there has been no evidence for their interaction with an equivalent invertebrate model. The results obtained in this investigation have identified a previously unknown target for volatile phenolic phytochemicals: the insect GABA receptor. Thymol, eugenol, and carvacrol all enhance the action of GABA on heterologously expressed, homomeric insect GABA receptors, composed of the *Drosophila melanogaster* subunit RDL<sub>ac</sub>. As thymol and carvacrol were shown to be amongst the most lethal of a selection of monoterpenoids towards human lice (*P. humanus*, see Chapter 2 of this thesis), and thymol, eugenol and carvacrol have all been shown to be lethal towards some other species of insects [157,197], low molecular weight phenolic phytochemicals could represent a new class of GABAergic insecticidal molecules.

Resistance to many neuroactive insecticides is widespread and may involve, for example, alterations in binding site or a change in metabolic aspects relating to a particular chemical structure, such as increased detoxification [27,86]. Cross-resistance can occur not only between structurally similar molecules, but also between chemically unrelated molecules that have a common mechanism of action or pharmacokinetic property, for example, the insecticidal molecules picrotoxinin, fipronil and BIDN are all rendered less effective by the A302S mutation in RDL subunits [39,88,113,114]; although they are structurally diverse, all are GABAR antagonists. If the insect GABA receptor proves to be the mediator of monoterpenoid- or eugenol-induced lethality in insects, provided that these compounds do not depend on A302 of RDL for their potentiatory activity, insects with the A302S mutation would not be cross-resistant to them. So far, the binding sites for GABAR positive allosteric modulators appear to be distinct from those of GABAR antagonists, and, as the A302S mutation is thought to reside in the binding site, rather than the transduction domain, of this particular group of antagonists, cross-resistance is unlikely.

Despite numerous reports of the insecticidal and insect behaviour-influencing nature of monoterpenoids and other volatile phytochemicals [101,135,196], there have been few attempts to relate these effects to insect molecular targets. This study provides the first evidence for the action of phenolic monoterpenoids and eugenol at a specific target in the insect nervous system, as it demonstrates their activity at a recombinant insect ionotropic receptor known to be representative of many native insect GABARs.

### **3.5.2 A putative role for GABA receptors in mediating the effects of monoterpenoids on insects**

The efficacy of the three volatile phytochemicals studied on RDL<sub>ac</sub> homomers, in order of decreasing potency, was: thymol > carvacrol > eugenol. In Chapter 2 of this thesis, thymol was also more potent than carvacrol at killing lice (the pediculicidal activity of eugenol has not yet been determined), suggesting there may be a correlation between the insecticidal activity of at least some monoterpenoids and the potency of insect RDL<sub>ac</sub> GABAR modulation. This could be further evidence that these essential oil components exert their lethal activity via potentiation of insect GABARs, but more data in each assay system is required to confirm the correlation. However, it appears that not all monoterpenoids, even the most pediculicidal ones, have potentiatory activity at GABARs; preliminary observations suggest that terpinen-4-ol and linalool have no effect on RDL<sub>ac</sub> homomers and that  $\alpha$ -thujone produces a slight inhibition, as it does on GABA<sub>A</sub> receptors [110]. It is not inconceivable that different monoterpenoids with similar insecticidal potencies may have very different mechanisms of action, and the present data suggests that even the relatively small structural variation that exists between monoterpenoids may result in different effects at the same target. Thus far, potentiation of GABAergic activity at insect GABA receptors appears to be common to the phenolic monoterpenoids, although citronellol, citronellal and pinene were reported to potentiate responses of rat ionotropic GABA receptors expressed in *Xenopus* oocytes [285]; furthermore, the relative potency of the phenolic essential oil constituents as pediculicides and also their effects on louse physiology, including reduction of locomotory activity suggestive of a CNS depressant effect, indicates that potentiation of GABA may underlie their lethality towards *P. humanus*.

Lack of activity at RDL<sub>ac</sub> homomers may not necessarily reflect absence of activity at native insect GABARs, as such compounds may require the presence of more than one subunit type to act, analogous to the way that benzodiazepines require the presence of  $\alpha$  and  $\gamma$

subunits to potentiate at mammalian GABARs, or they may act specifically on certain populations of GABA receptors. The reason for the species and developmental stage specificity described for insecticidal monoterpenoids [196] is as yet undetermined, it may reflect differences in GABA receptor populations, but other factors such as differences in bioavailability of the active moiety at the target site are equally likely.

Future studies could include the characterisation of the  $\alpha$ -thujone inhibitory effect on  $RDL_{ac}$ , and to investigate if the binding site is shared with thymol, eugenol and carvacrol; although they have similar physicochemical properties, it does not follow that their site of action on GABARs is the same. Even the two isomers of HCH,  $\gamma$  (GABA-blocking) and  $\delta$  (GABA-potentiating), are believed to have different binding sites, as the A302S mutation reduces the potency of the former but not the latter in  $RDL_{ac}$  homomers [23].

### ***3.5.3 How does the activity of thymol, eugenol and carvacrol in potentiating GABA-mediated currents compare with the actions of other insecticides?***

Several classes of insecticidal molecules have activity on the invertebrate nervous system: Some insecticides enhance neuronal activity by acting as agonists at receptors mediating excitatory stimuli. For example, pyrethroids maintain insect voltage-gated  $Na^+$ -channels in the open state for extended periods [277], and the neonicotinoids, such as the nitroguanidine imidacloprid, are agonists at nAChRs [78]. The organophosphates result in neuronal stimulation by inhibiting the break-down of the excitatory transmitter ACh. Others insecticides enhance overall neuronal activity by blocking GABA-mediated inhibition in the nervous system [78]. Such convulsant antagonists with insecticidal activity include picrotoxin, cyclodienes, hexachlorocyclohexanes, BIDN and fipronil, all of which result in a net over-stimulation of the nervous system by decreasing the inhibitory input.

Unfortunately, cross-resistance to GABAergic insecticides is extensive, and not only affects structurally diverse molecules, but also affects ligands of more than one binding site: Radioligand binding and electrophysiological studies suggest that several convulsant antagonist insecticides, such as BIDN and dieldrin, interact with a common binding site yet fipronil and BIDN, and BIDN and picrotoxin do not share common binding locations [113,208]. Despite this, the potency of all of these compounds is reduced by the A302S mutation in *Drosophila Rdl*-encoded subunits and therefore the amino acid substitution is clearly not in an area responsible for transducing the effects of a single chemical class. Thus

the A302S mutation may either be within an area overlapped by the binding domains of several classes of antagonist, or it may affect common conformational changes induced or prevented by them. Monoterpenoids and eugenol are smaller than most antagonist compounds, and therefore probably have more discrete binding location(s). As the effects of potentiating compounds and blockers on channel activity are different it also follows that the conformational changes they influence, and the residues involved, are also distinct. This theory is supported by the differential effect of the A302S mutation on  $\delta$ - and  $\gamma$ -HCH activity at RDL<sub>ac</sub> homomers.

The fact that the vast majority of insecticides act to increase insect neuronal activity may seem incompatible with the theory that the toxic actions of some monoterpenoids are dependent on their GABA-enhancing action, as this activity would lead to neuronal inhibition. However, a few other insect-lethal agents have been discovered which decrease neuronal activity, for example, the oxadiazines are insecticidal towards a variety of lepidopteran pests and act by blocking sodium dependent action-potentials [166]. A recent study on the dorsal unpaired median (DUM) neurones of *P. americana* revealed spontaneous action potential inhibition by DCJW (the active metabolite of the oxadiazine Indoxacarb) via block of both voltage-dependent sodium channels involved in the depolarisation phase of the action potential (those targeted by pyrethroids), and also so-called background Na<sup>+</sup> channels involved in maintaining the resting  $E_m$  and driving  $E_m$  towards the threshold for action potential generation [145].

The fact that oxadiazine-induced reduction of spontaneous action potential amplitude via voltage-dependent Na<sup>+</sup>-channel block proves lethal to insects supports the theory that phenolic monoterpenoids are insecticidal via GABA-potentiation, as decreased action potential amplitude is also a potential outcome of increased GABA activity.

#### **3.5.4 Are other molecules which potentiate insect GABA activity insecticidal?**

Many compounds, such as barbiturates, steroids and propofol, have all been found to potentiate the effect of GABA at native insect receptors and at RDL<sub>ac</sub> homomers. Unlike monoterpenoids and eugenol, which have been reported to be lethal towards certain species, these compounds are not generally considered insecticidal,  $\delta$ -HCH being the only exception. This observation does not preclude potentiation of GABA activity as an insecticidal mechanism; there are many determinants of insecticidal activity, such as

cuticular penetration, tissue distribution and metabolism, not just potency at the site of action [78].

It is also possible that the maximum degree of enhancement produced by GABA-potentiating compounds at insect receptors may determine lethality. Table 3.1 lists the maximal potentiations generated by a variety of modulatory compounds at  $RDL_{ac}$  receptors; of the compounds tested in previous studies,  $\delta$ -HCH gives the largest maximal potentiation and it is also the only compound in the group with renowned insecticidal activity. Thymol shows a greater degree of potentiation than carvacrol over the range  $10^{-5}$  M to  $10^{-4}$  M, and was also found to be more insecticidal towards lice in Chapter 2 of this thesis. Although eugenol has not been tested on lice, it was found to be insecticidal towards oak nut weevil, *Mechoris ursulus* [197]. It is difficult to compare the figures shown here for thymol, eugenol and carvacrol and other allosterically active agents, as percentage enhancements of  $EC_{20}$  concentrations are lower than those of  $EC_{10}$  doses (refer to [116] and also Chapter 4 of this thesis for illustrative dose-response curves). Furthermore, in contrast to the other modulatory compounds, it was not possible to obtain maximal enhancements for thymol, eugenol and carvacrol. However, as the maximum potentiation values for monoterpenoids and eugenol shown in Table 3.1 may be substantial underestimates of the true values, it may be worth investigating the link between maximal enhancement values and insecticidal activity.

Another important difference between thymol, eugenol, carvacrol, and the steroids, barbiturates, and propofol is that the second group of compounds all have direct agonist activity at mammalian  $GABA_A$  receptors, but none tested so far have this property at  $RDL_{ac}$  homomers. By contrast, thymol clearly has direct activity at both mammalian [176] and  $RDL_{ac}$  receptors. As some authors believe that it may be the direct GABA receptor agonist effect of anaesthetics that is responsible for their activity, and overdoses of these compounds are lethal to humans, perhaps the equivalent activity in insects may also be responsible for the insecticidal effect.

It is interesting that a previous study of the actions of HCH isomers at GABA receptors did not relate their activity to GABAR block or potentiation per se, but instead found a correlation between insecticidal potency and the ability of the isomer to enhance receptor desensitisation [181]. Enhancing GABAR desensitisation would have the effect of rendering GABAergic neurotransmission unresponsive to endogenous GABA, allowing the nervous system to become overstimulated. The potential of volatile phenolic

phytochemicals in influencing the rate of receptor desensitisation in the presence of GABA has not yet been tested, however, in this study, agonist responses induced by thymol did not desensitise over the 2 min application period. This may have been because only low concentrations on the agonist dose-response curve were tested, as only those responses to GABA above EC<sub>20</sub> show marked desensitisation. However, non-desensitising agonist responses to EC<sub>100</sub> thymol were reported in rat  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors [176]. If receptor desensitisation proves to be a feature of monoterpenoid action it is likely to confuse studies of physiological responses in whole insects, as it is likely that evidence of both neuronal suppression and excitation would be observed prior to death.

Because of the multitude of determining factors, correlations between insecticidal potency and GABAergic activity made in this thesis and in other work must be treated purely as hypotheses. Further investigation is required to confirm the link between the GABA-ergic and insecticidal activities of volatile phytochemicals.

	Maximal enhancement (%)	GABA conc <sup>n</sup>	References
Eugenol	1713	EC <sub>20</sub>	Chapter 3
Thymol	715	EC <sub>20</sub>	Chapter 3
Carvacrol	362	EC <sub>20</sub>	Chapter 3
$\delta$ -HCH	1000	EC <sub>10</sub>	[24]
Propofol	880	EC <sub>10</sub>	[24]
Pentobarbitone	790	EC <sub>10</sub>	[24]
5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one	180	EC <sub>10</sub>	[24]
4'-chlordiazepam	180	EC <sub>10</sub>	[116]

Control response to GABA = 100 %

**Table 3.1 Allosteric modulation of RDL homomers**

A comparison of the maximal degrees of enhancement of GABA EC<sub>10</sub> and EC<sub>20</sub> concentrations by various allosteric modulators on homomers composed of RDL<sub>ac</sub>. Conc<sup>n</sup> = concentration.



### ***Summary and direction***

In Chapter 3, the ability of the volatile phenolic essential oil constituents thymol, eugenol and carvacrol to enhance the actions of GABA at heterologously expressed RDL<sub>ac</sub> homomers was demonstrated. These homomeric multi-subunit proteins are a representative model of a major type of insect GABA receptor, in that they reliably reproduce the pharmacology of many insect receptors studied in the native tissue.

In Chapter 4, the question of whether one of these compounds, thymol, has similar effects at a human ionotropic GABA receptor model will be addressed. If this proves to be the case, the fact that human GABA receptors have been more thoroughly studied than their insect counterparts, and have a wider range of associated pharmacological and molecular tools, means they will make a more useful vehicle than the RDL homomer for the Chapter 4 study of the thymol binding site.

## Chapter 4

### Action of monoterpenoids on mammalian GABA receptors

#### 4.1 Rationale for investigating monoterpene activity on mammalian GABA receptors

The purpose of the experiments described in this chapter was to investigate whether the positive modulation of GABA action by thymol on recombinant RDL<sub>ac</sub>-containing insect receptors was also apparent on vertebrate receptors and, if so, whether thymol was acting on a previously uncharacterised site of action on the GABA receptor. Selectivity for the invertebrate receptor would be toxicologically advantageous if thymol were to be developed as an insecticide. Conversely, activity at both insect GABARs and mammalian GABA<sub>A</sub>Rs would allow further investigation of the site of action, as the multiple subunits available and more potent positive modulatory ligands make the GABA<sub>A</sub>R a more useful tool for this purpose than the insect GABAR.

Previous literature has concluded that potentiating compounds, such as barbiturates, steroids, and benzodiazepines, act at GABA receptors via an allosteric site, unrelated to either the agonist or convulsant antagonist binding sites [241]. In this Chapter, tests were devised to screen for the interaction of thymol with one of the various allosteric modulator binding sites.

Interactions of ligands with particular sites on neuronal receptors can be analysed in binding studies [see for example 192,193], or by electrophysiology. Electrophysiological experiments utilising recombinant receptors of known subunit composition are the most informative: By changing the subunit combination of heteromeric receptors, the subunit dependency of ligands such as the benzodiazepines and loreclezole can be identified; alternatively, by keeping the same subunit combination, the effects of combinations of ligands can be observed to investigate if they compete for the same binding site; another approach is to generate mutant subunits, often using either chimaeric or point-mutated gene sequences, to assess the dependency of ligand action on certain sections of the protein or individual amino acids. The latter experiment can be problematic, for example, a major

drawback is that not all mutant proteins express as well as wild-type subunits. A common approach is to observe differential efficacy of a compound on receptors varying in one subunit type, identify the residues in which the two subunit types differ, and systematically mutate runs of these residues in the sensitive subunit to residues in the insensitive subunit, until sensitivity is abolished (or vice versa); then the number of residues mutated are reduced until the minimum structural requirement(s) is identified. This allows the researcher to narrow down potential areas for site-directed mutagenesis.

## **4.2 Methods for expression of recombinant human GABA receptors**

### **4.2.1 Oocyte preparation and cDNA Injection**

Ovaries from mature *Xenopus laevis* females (Blades, U.K.) were provided by Ms Sally Thompson and Ms Lola Wheat at Merck, Sharp and Dohme, Terling's Park, U.K. The ovaries were immersed in Modified Barth's Solution (MBS) of the following constitution (mM): NaCl, 88; KCl, 1; NaHCO<sub>3</sub>, 2.4; HEPES, 10; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.82; Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 0.33; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.91; pH 7.5 by addition of 10 N and/or 1 N NaOH. The ovary tissue was divided into groups of 100-200 oocytes using fine forceps and transferred to a hypertonic isolation medium composed of (mM): NaCl, 108; KCl, 2; EDTA, 1.2; HEPES, 10; pH 7.9 with NaOH. In this medium the oocytes shrank away from the surrounding follicular membranes, facilitating manual membrane detachment with forceps. Residual follicular cells were removed by incubating the oocytes in collagenase type IA (Sigma, U.K.), 0.5 mg/ml in MBS, for 6 min.

Each cell nucleus was injected with 20 nl of GABA subunit cDNA mixture using a manual oocyte injection pipette (Drummond, U.K.). After injection, cells were transferred to individual compartments of a multiwell plate, each of which contained 2 ml of incubation medium. The incubation medium in this case was MBS supplemented with gentamycin, 50 mg/l; penicillin, 10,000 U/l; streptomycin, 10 mg/l; and sodium pyruvate, 2.5 mM. Oocytes were maintained at 19 °C initially, but were transferred to 4 °C if necessary to keep expression levels in check; over-expression can lead to perturbations in subunit stoichiometry away from the 2 $\alpha$ 2 $\beta$ 1 $\gamma$  ratio desired.

### **GABA subunit cDNAs**

Human cDNAs, encoding  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 3$ ,  $\beta 1$  and  $\gamma 2s$  GABA receptor subunits, were supplied by The Molecular Biology Department, Merck, Sharp and Dohme, Terling's Park, U.K. The subunit cDNAs were injected in combinations of three, in the ratios 1:1:1 or 1:0.1:1 ( $\alpha$ ,  $\beta$ ,  $\gamma$ , respectively), the concentration of total cDNA being 20 ng/ml in injection buffer comprising of (mM): NaCl, 88; KCl, 1; HEPES, 15; pH 7 with NaOH.

### **4.2.2 Electrophysiology**

Recordings were obtained from cells as soon as sufficient expression was detected, usually 18-24 h after injection. Cells were only used if maximal responses of 100 nA or more could be elicited by applications of 3 mM GABA ( $EC_{100}$ ). Oocytes were secured inside a ring of stainless steel entomological pins embedded into Sylgard, which lined the bottom of a 400  $\mu$ l perspex bath. Fresh MBS was continually perfused through the chamber by a gravity fed system at 4 ml/min. All drugs were applied dissolved in MBS, although stock solutions of hydrophobic compounds were prepared in DMSO or acetone for further dilution in saline. Membrane currents were measured by two-electrode voltage-clamp, with the membrane held at -60 mV, using 2 M KCl-filled electrodes with 1 % agar in 2 M KCl at the tip. Electrode resistance was maintained at 0.5-5 M $\Omega$ . Signals were amplified using a GeneClamp 500 (Axon Instruments, U.S.A.) and recorded on two outputs in tandem: electronically using 'Oocyte' for the DigiTimer Digistore™ System (DigiTimer Ltd., U.K.) and on chart paper with a Thermal Arraycorder WR 8500 series (Graphtec, U.K.).

Each cell was first challenged with 3 mM GABA to obtain the maximal response, then GABA dose-response curves or isolated  $EC_{20}s$  were obtained prior to the assessment of thymol action in the same cell. By applying increasing GABA or thymol concentrations, dose-response data were generated. In order to minimise desensitisation and run-down effects, oocytes were challenged with drugs 5 min after a maximal GABA response, and 3-7 min after other applications, depending on the drug and concentration applied. Only oocytes yielding stable responses were selected for experimental work. Uninjected oocytes did not respond to GABA. Curves were fitted to the dose-response data, both for individual cells and also to the mean data points, using the non-linear regression equation in GraphPad Prism described in Section 3.3.2. Results are presented as the mean  $\pm$  one s.e.m.  $EC_{50}$  values given in the text are mean values calculated from several  $EC_{50}$  values,

each of which was estimated from the dose-response data obtained from an individual cell. For the graphical presentation of data, all dose-response results were averaged before a single regression line was fitted.

### **Chemicals**

Acetone, 99.8 %, was obtained from BDH; DMSO, 99.99 %, from Fisher Scientific; 3-HMC from Tocris and propofol (2,6-diisopropylphenol) from Aldrich. GABA, pentobarbital, 5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol, 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (allopregnanolone) and thymol were all supplied by Sigma (U.K.). Flumazenil (Ro151788) was synthesised by K. Moore at The Chemistry Department, Merck, Sharp and Dohme, Terling's Park, U.K.

## **4.3 Results**

### **4.3.1 Potentiation of GABA action by thymol**

Thymol, 1  $\mu$ M - 100  $\mu$ M, applied in conjunction with EC<sub>20</sub> GABA produced a dose-dependent potentiation of the GABA response in oocytes injected with  $\alpha$ 1 $\beta$ 3 $\gamma$ 2s cDNAs (Figure 4.1 (a) and (b) and Figure 4.2). Above 100  $\mu$ M, potentiation decreased with increasing thymol concentration (Figure 4.1 (c)). The maximal potentiation achieved was  $416 \pm 72$  %, at 100  $\mu$ M thymol ( $n = 5$ ).

### **4.3.2 Intrinsic activity of thymol**

Cells expressing  $\alpha$ 1 $\beta$ 3 $\gamma$ 2s did not respond to 1  $\mu$ M-50  $\mu$ M thymol, when applied alone (any changes in clamping current were less than 9 nA in amplitude). Thymol at 100  $\mu$ M and above generated responses, although these were extremely small in comparison to the 100  $\mu$ M thymol-potentiated GABA current (Figure 4.1 (b) and (c): Responses to 100  $\mu$ M thymol measured an average of 9 nA ( $n = 7$ ), in the same cells the mean potentiated GABA current, measured from the baseline, was 878 nA. Dose-response curves showing the agonist action of thymol were not examined because the concentrations of acetone required to solubilise 300  $\mu$ M thymol and above have increasing non-specific effects on the current required to clamp the cell.

#### **4.3.3 Effect of thymol on the GABA dose-response curve**

Thymol enhances the response to concentrations of GABA below  $EC_{100}$ , the largest effects being seen over the range  $EC_5 - EC_{50}$  (Figure 4.3). The amplitudes of currents induced by 3  $\mu$ M and 100  $\mu$ M GABA were  $7.6 \pm 1.7 \%$  ( $n = 5$ ) and  $89.0 \pm 4.8 \%$  ( $n = 5$ ) of the maximum, respectively. In the presence of 50  $\mu$ M thymol, these responses were potentiated to  $45.0 \pm 5.2 \%$  ( $n = 5$ ) and  $98.6 \pm 3.0 \%$  ( $n = 5$ ) of the maximum GABA response, respectively. The GABA dose-response curve, (calculated by non-linear regression analysis of the mean data points from 5 cells) was shifted to the left by 50  $\mu$ M thymol and the mean  $EC_{50}$  for GABA (the average of the  $EC_{50}$ s from five individual dose-response curves) was significantly reduced from  $15 \pm 3 \mu$ M to  $4 \pm 1 \mu$ M, as demonstrated by a paired t-test ( $p = 0.0044$ ) at the  $p = 0.05$  level. The Hill slope was also reduced significantly (shown by a paired t-test at the  $p = 0.05$  level, with a  $p$  value of 0.0076) from  $1.35 \pm 0.14$  to  $1.04 \pm 0.16$ . However, 50  $\mu$ M thymol had little effect on the maximum GABA response (Figure 4.2).

#### **4.3.4 Effect of different subunit compositions on thymol potentiation**

Over the range 1  $\mu$ M-100  $\mu$ M, thymol potentiated the  $EC_{20}$  GABA response mediated by  $\alpha 1\beta 1\gamma 2$ s and  $\alpha 6\beta 3\gamma 2$ s vertebrate GABA receptors in a dose-dependent manner. The thymol dose-response curves obtained for  $\alpha 1\beta 1\gamma 2$ s,  $\alpha 6\beta 3\gamma 2$ s and  $\alpha 1\beta 3\gamma 2$ s receptors were almost identical: over the range of concentrations tested, GABA activity at all of these three subunit combinations was potentiated equally by thymol (Figures 4.4 and 4.5).

#### **4.3.5 Effect of the benzodiazepine non-competitive GABA antagonist flumazenil, on thymol potentiation**

Application of 50  $\mu$ M thymol,  $EC_{20}$  GABA and 1  $\mu$ M flumazenil to  $\alpha 1\beta 3\gamma 2$ s expressing oocytes following pre-applications of flumazenil (30 s) and thymol plus flumazenil (40 s) resulted in a mean increase of the  $EC_{20}$  GABA response to  $276.3 \pm 6.6 \%$  ( $n = 4$ ) (Figure 4.6 (a) and (c)). Co-application of thymol and GABA, following a 40 s pre-incubation with thymol, potentiated the GABA response to  $346.5 \pm 34.7 \%$  ( $n = 4$ ) (Figure 4.6 (b)). These two sets of data were obtained from different oocytes, so an unpaired t-test was performed

to examine the flumazenil effect. The mean of the data for thymol potentiation in the presence of 1  $\mu\text{M}$  flumazenil was found not to be significantly different from that in its absence at the  $p = 0.05$  level ( $p$  was 0.0936), suggesting thymol is not acting via the benzodiazepine site

#### **4.3.6 Effect of 3-hydroxymethyl- $\beta$ -carboline (3-HMC) on thymol potentiation**

In cells expressing  $\alpha 1\beta 3\gamma 2\text{s}$ , a slight decrease in the  $\text{EC}_{20}$  GABA response was apparent in the presence of 100  $\mu\text{M}$  3-HMC, the amplitude being  $83.7 \pm 3 \%$  ( $n = 3$ ) of control (Figure 4.7). The oocytes were then challenged with 50  $\mu\text{M}$  thymol and  $\text{EC}_{20}$  GABA in combination, which resulted in potentiation of the control GABA response to  $320.3 \pm 60 \%$  ( $n = 3$ ). Application of 50  $\mu\text{M}$  thymol,  $\text{EC}_{20}$  GABA and 100  $\mu\text{M}$  3-HMC together, following pre-applications of thymol and 3-HMC, resulted in an augmentation of the  $\text{EC}_{20}$  GABA response to  $308 \pm 64 \%$  ( $n = 3$ ). The difference between the normalised, thymol-potentiated  $\text{EC}_{20}$  GABA response data in the presence and absence of 3-HMC was shown to be non-significant at the  $p = 0.05$  level ( $p = 0.1264$ ) in a paired t-test.

#### **4.3.7 Effect of the pregnane steroid 5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol on thymol potentiation**

Co-application of the pregnane steroid 5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol, 3  $\mu\text{M}$ , with  $\text{EC}_{20}$  GABA increased the  $\alpha 1\beta 3\gamma 2\text{s}$  GABA response to  $164 \pm 26 \%$  ( $n = 4$ ) of control (Figure 4.8). The mean potentiation of the  $\text{EC}_{20}$  GABA response by 50  $\mu\text{M}$  thymol in these cells was  $360 \pm 37 \%$  ( $n = 4$ ) of control. Application of thymol, GABA and 5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol, following pre-applications of 5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol alone (40 s) then thymol plus 5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol (40 s) enhanced the control response to  $395 \pm 39 \%$  ( $n = 4$ ). A paired t-test demonstrated that  $\text{EC}_{20}$  GABA potentiation by 50  $\mu\text{M}$  thymol was significantly larger than that of 3 $\mu\text{M}$  5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol at the  $p = 0.05$  level ( $p$  was 0.0256). A further paired t-test demonstrated that 3 $\mu\text{M}$  5 $\alpha$ -pregnane-3 $\alpha$ , 20 $\alpha$ -diol served to significantly augment the response to co-applied thymol and GABA at the  $p = 0.05$  level ( $p$  was 0.0287).

#### **4.3.8 Effect of the barbiturate pentobarbital on thymol potentiation**

Pentobarbital elicited inward currents in oocytes expressing  $\alpha 1\beta 3\gamma 2s$  (Figure 4.9). Repeated doses of 300  $\mu\text{M}$  pentobarbital were applied until consistent responses were obtained, the mean of which was  $558 \pm 315 \text{ nA}$  ( $n = 4$ ). Thymol, 50  $\mu\text{M}$ , was then applied for 40 s prior to co-application of thymol with 300  $\mu\text{M}$  pentobarbital. The response to pentobarbital was potentiated by 50  $\mu\text{M}$  thymol to  $996 \pm 567 \%$  ( $n = 4$ ). A paired, one-tailed t-test of the results, expressed as a percentage of the pentobarbital current, showed the potentiation to be significant at the  $p = 0.05$  level ( $p$  was 0.0263).

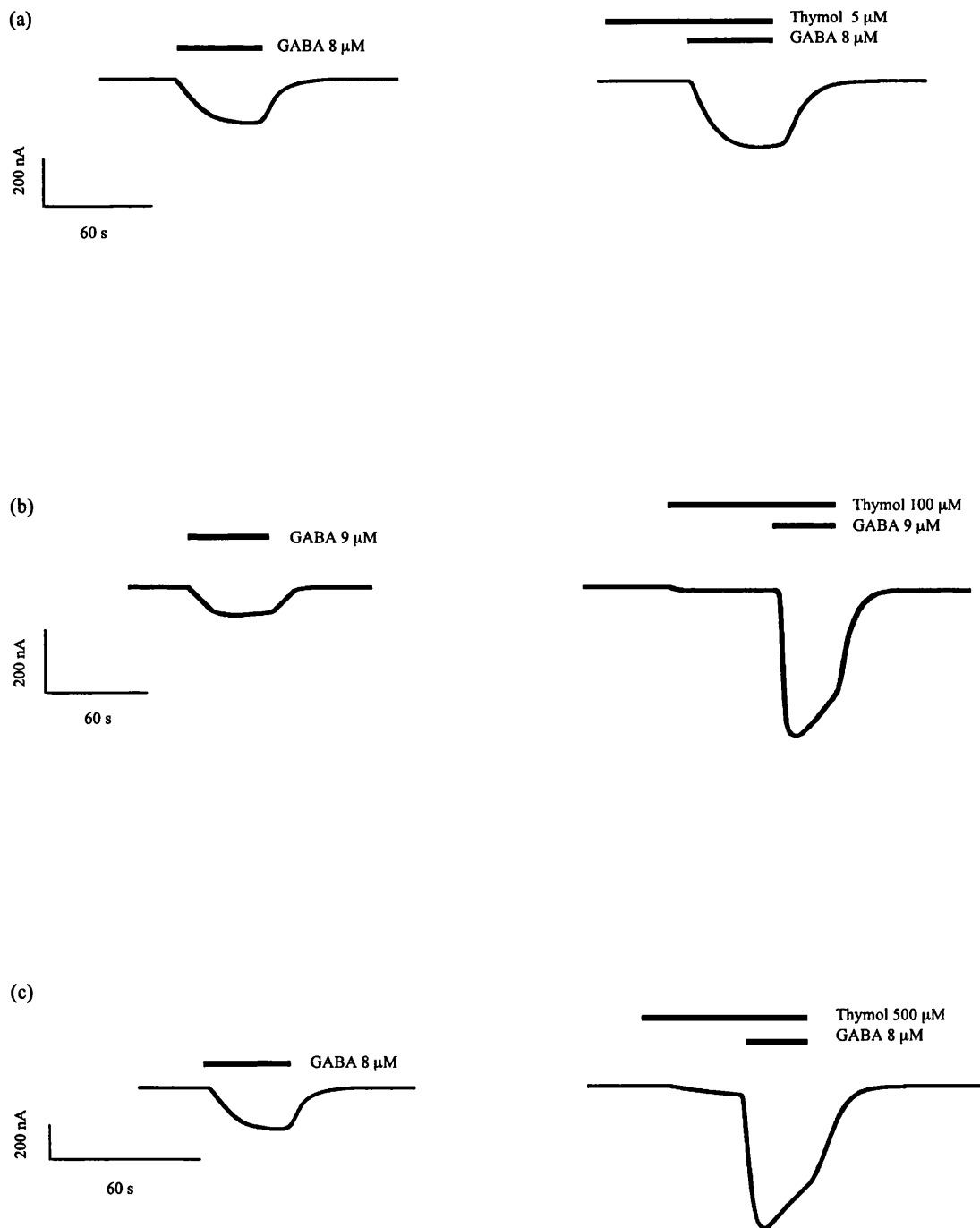


#### **4.3.9 Effect of the general anaesthetic propofol on thymol potentiation**

Oocytes expressing  $\alpha 1\beta 3\gamma 2s$  responded to applications of 60  $\mu M$  propofol with inward currents (Figures 4.10 and 4.11). Propofol was applied until consistent responses, with a mean of  $1236 \pm 87$  nA ( $n = 4$ ) were obtained. Thymol, 50  $\mu M$ , was applied for 40 s prior to co-application with 60  $\mu M$  propofol. The response to propofol was enhanced in the presence of thymol to  $1464 \pm 111$  % ( $n = 4$ ). A paired, one-tailed t-test of the results, when expressed as a percentage of the propofol current, showed the difference in means to be significant at the  $p = 0.05$  level ( $p$  was 0.0043).

As the potentiation achieved here was marginal, further tests were carried out: Firstly, 300 nM 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (allopregnanolone) was applied for 40 s followed by co-application of 300 nM allopregnanolone and 60  $\mu M$  propofol. Allopregnanolone alone generated a small current, 8.9 % ( $n = 1$ ) of the value of the mean control propofol response, and potentiated the current produced by 60  $\mu M$  propofol to 151 % ( $n = 1$ ). The purpose of applying allopregnanolone, a GABA-potentiating steroid, was to investigate the degree to which the 60  $\mu M$  propofol response could be potentiated by another positive modulator, known to act at a different site from propofol, and compare it with thymol potentiation of the 60  $\mu M$  propofol response in the same cell. Potentiation of the 60  $\mu M$  propofol response by thymol in this cell was 124 % of control ( $n = 1$ ).

In the same cell, a subsequent test compared thymol-induced potentiation of the propofol and GABA responses, to assess if the potentiation of GABA was greater. The response to 60  $\mu M$  propofol was found to approximate that of GABA  $EC_{30}$  in this cell and therefore the 50  $\mu M$  thymol-induced potentiation of GABA  $EC_{30}$  was assayed; it was found to be 197 % of control ( $n = 1$ ).



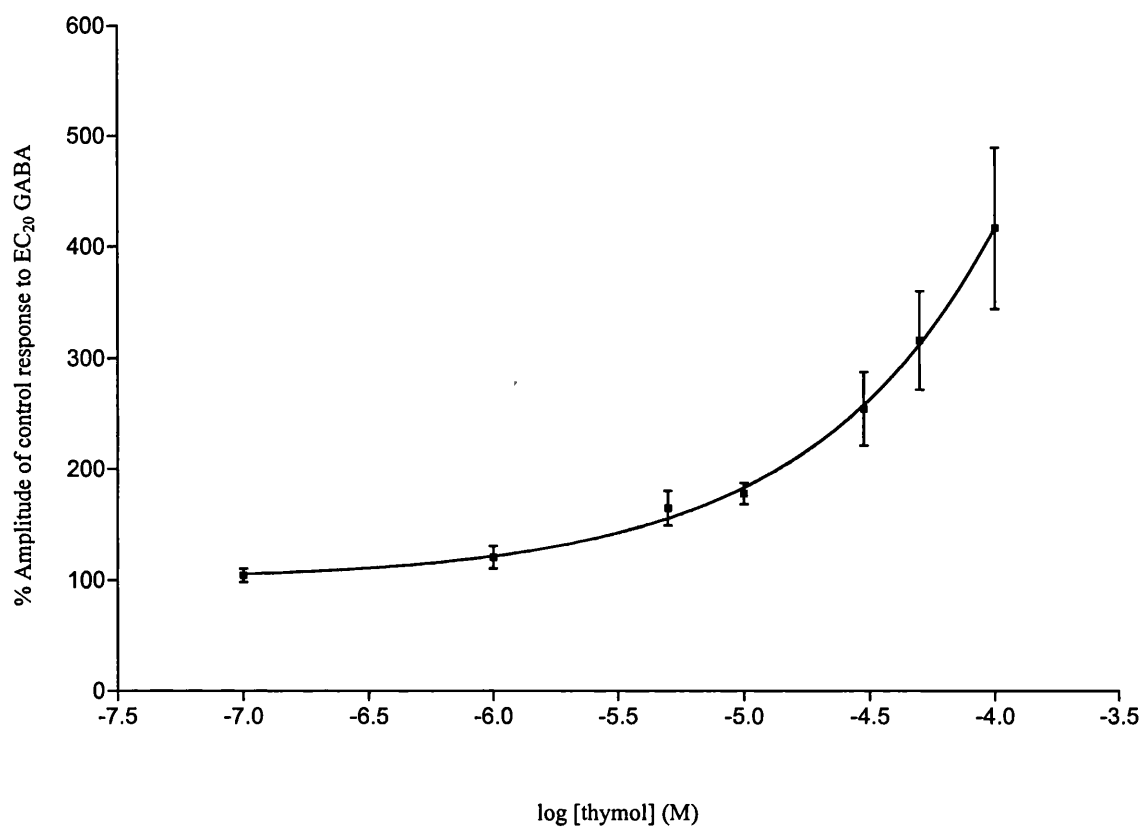
**Figure 4.1 Thymol-induced potentiation of submaximal GABA responses, recorded from heterologously expressed human GABA<sub>A</sub> receptors**

Recordings obtained from *Xenopus* oocytes expressing  $\alpha 1\beta 3\gamma 2s$  GABA receptor subunits, voltage-clamped at  $-60$  mV.

In (a), (b) and (c), traces demonstrating potentiation are shown adjacent to EC<sub>20</sub> GABA control responses recorded from the same cell.

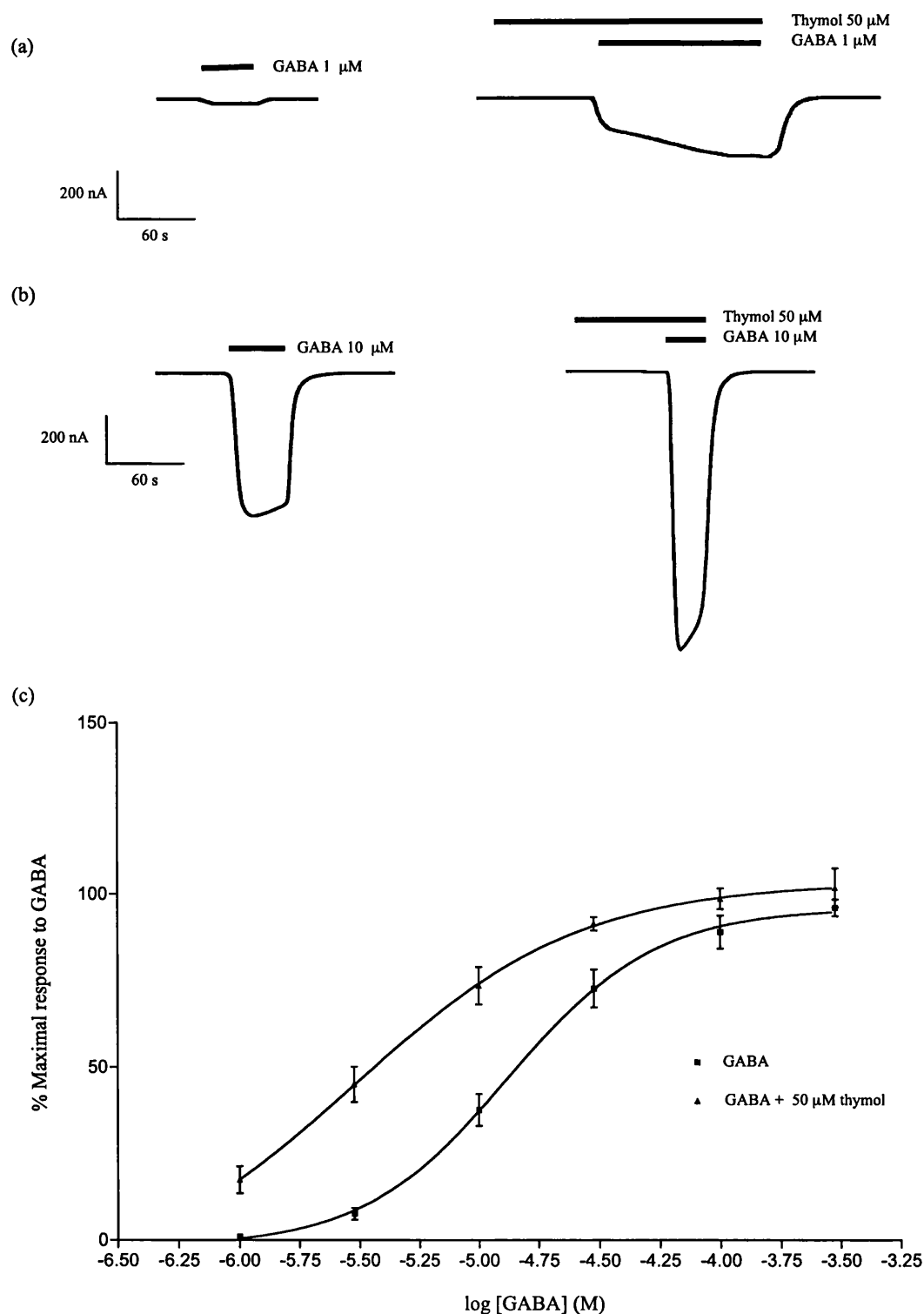
(a) Lower test concentrations of thymol, such as 5  $\mu$ M, had a smaller potentiating effect on the GABA response than (b) higher concentrations. Thymol, 100  $\mu$ M, induced a  $416 \pm 72\%$  ( $n = 5$ ) increase in the control GABA current, and also showed agonist activity when applied alone.

(c) Above 100  $\mu$ M, increasing thymol concentrations led to a decrease in the amplitude of the potentiated current and an increase in the agonist activity of thymol.



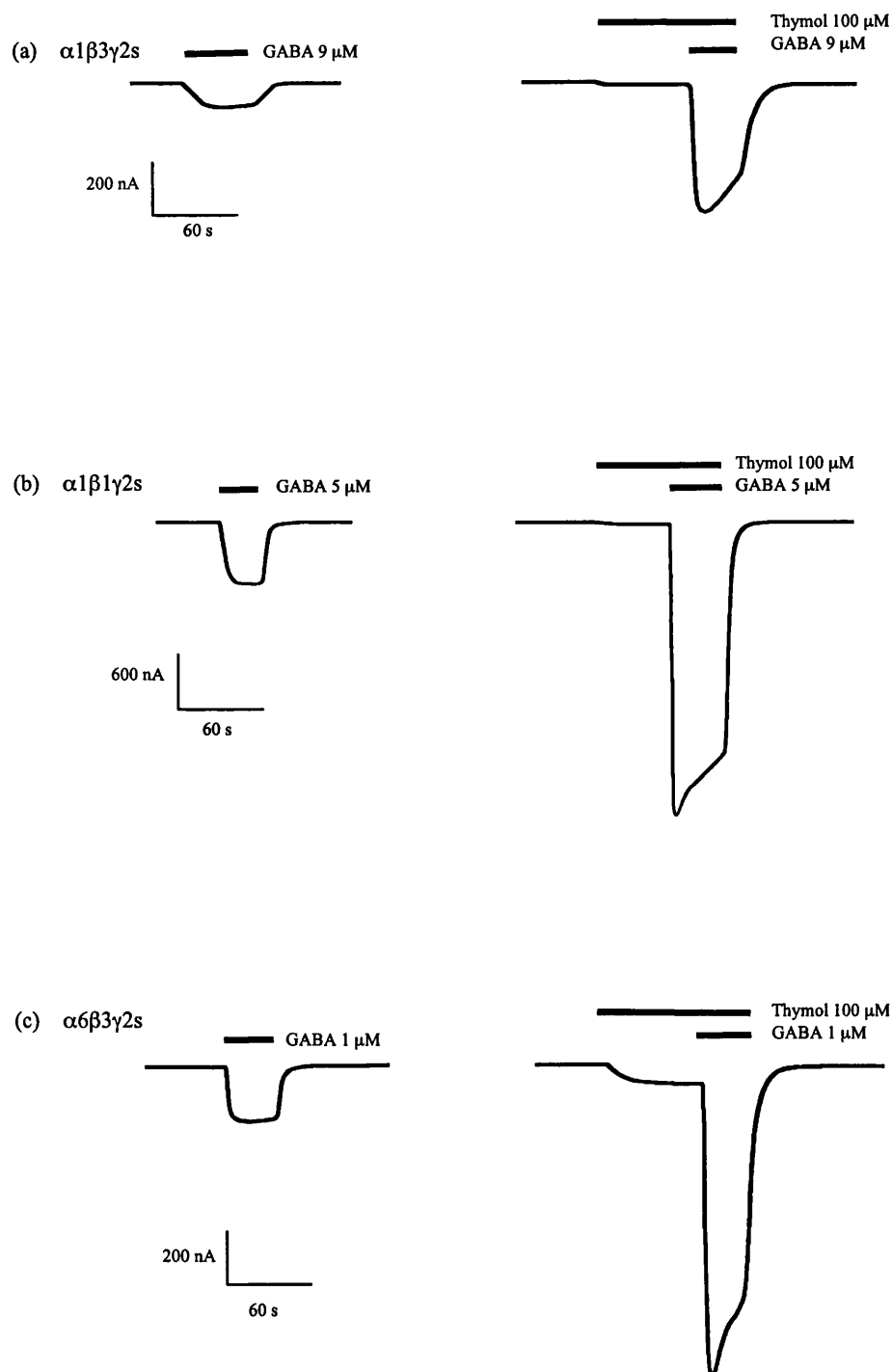
**Figure 4.2 Dose-dependent potentiation of the response to EC<sub>20</sub> GABA by thymol in *Xenopus* oocytes expressing human  $\alpha 1\beta 3\gamma 2s$  GABA<sub>A</sub> receptors**

The oocyte membrane potential was clamped at  $-60$  mV and potentiated currents recorded. Data are shown as the mean of 4-6 observations  $\pm$  one s.e.m.



**Figure 4.3 Effects of thymol on the GABA dose-response relationship of a human GABA<sub>A</sub> receptor**

(a) and (b): the degree of potentiation of the  $\alpha 1\beta 3\gamma 2\text{s}$  GABA response by 50  $\mu\text{M}$  thymol is [GABA]-dependent, and is greater when applied with lower test [GABA]. (c): Thymol, 50  $\mu\text{M}$  caused a significant leftwards shift of the GABA dose-response curve (at the  $p=0.05$  level). Data were normalised by expression as a percentage of the maximal GABA response. Data are shown as the mean of 5 observations  $\pm$  one s.e.m.

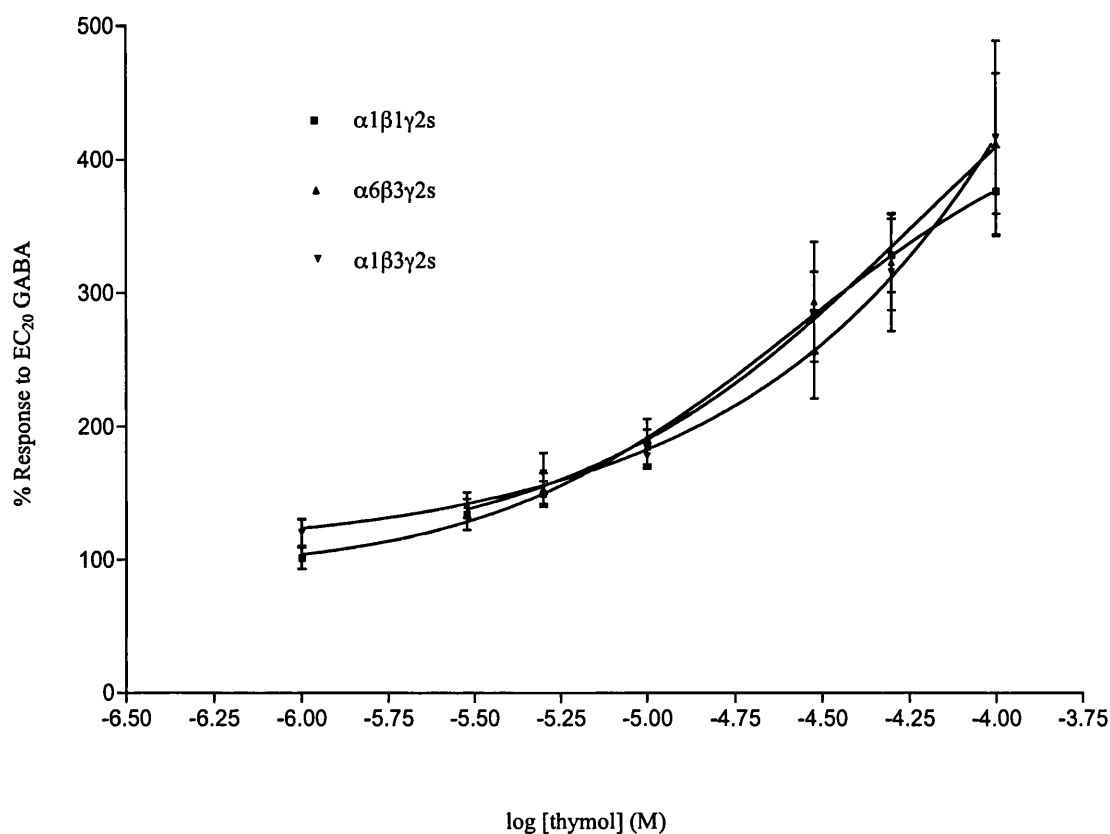


**Figure 4.4 The effects of thymol on the GABA responses of three different heterologously-expressed vertebrate GABA<sub>A</sub> receptors were not significantly different**

Thymol potentiates GABA at:

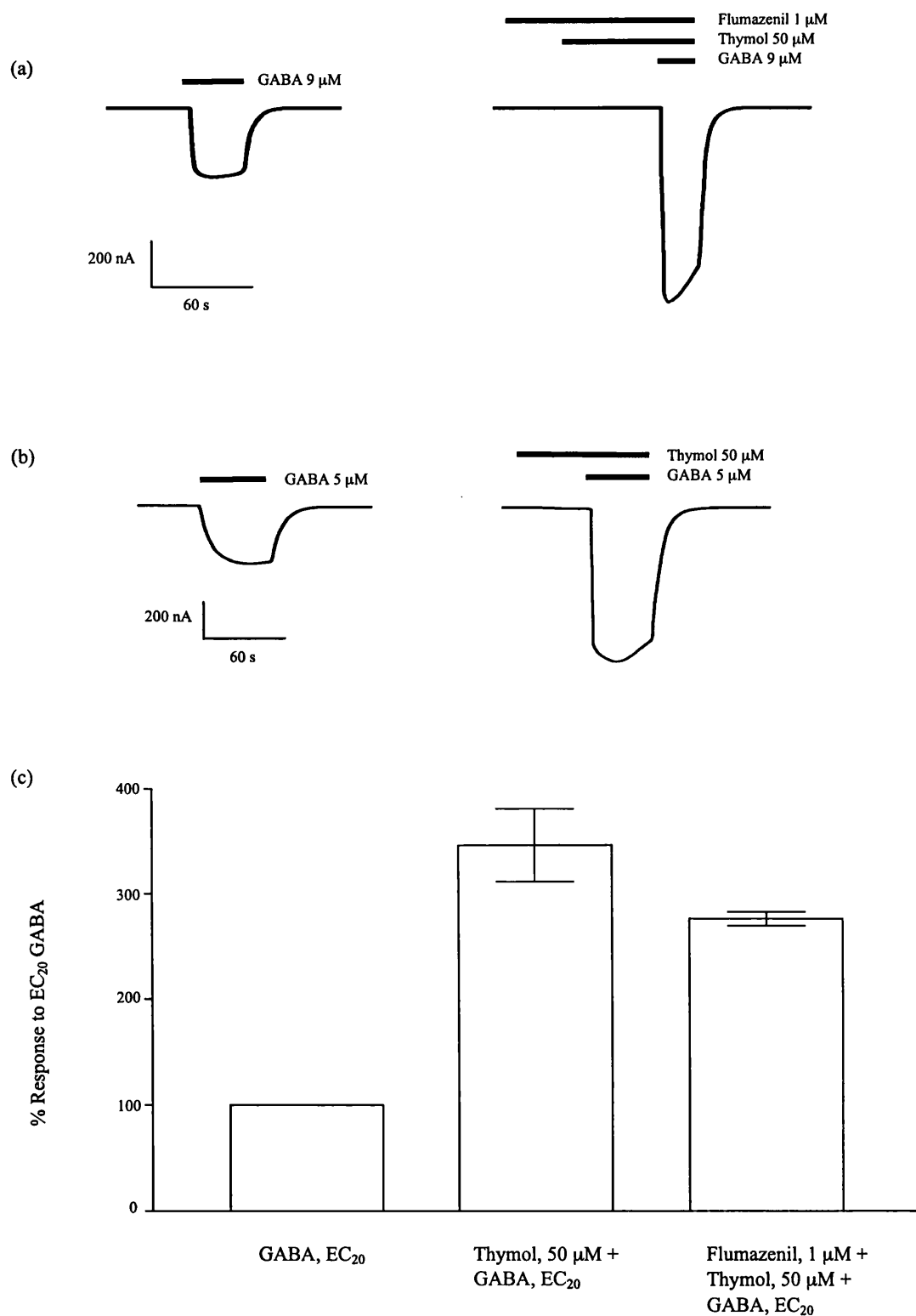
- (a)  $\alpha 1\beta 3\gamma 2s$  receptors (shown previously in Figure 4.1).
- (b)  $\alpha 1\beta 1\gamma 2s$  receptors
- (c)  $\alpha 6\beta 3\gamma 2s$  receptors

The above are shown alongside the control current elicited by EC<sub>20</sub> GABA, obtained in the same cell.



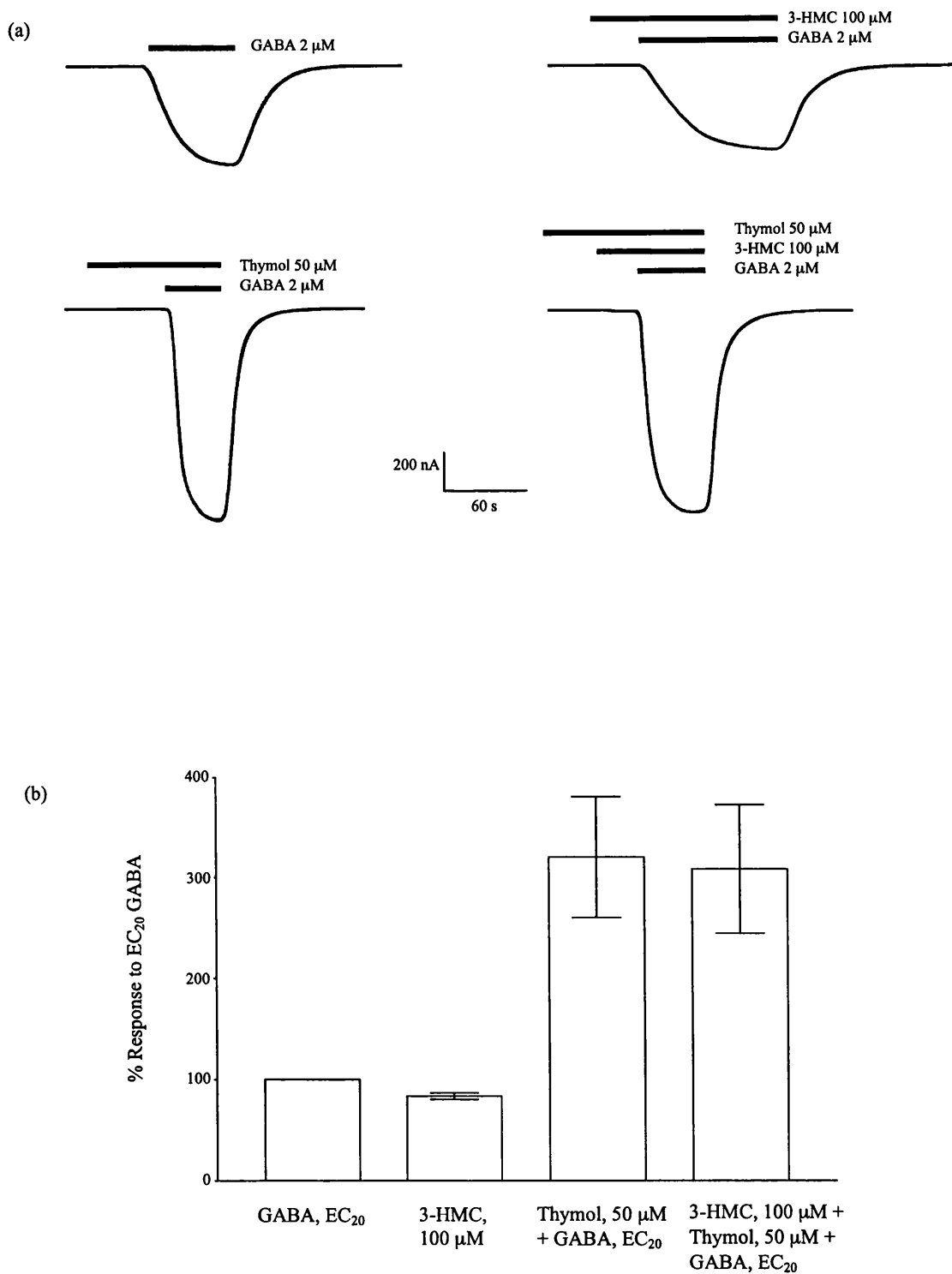
**Figure 4.5 The potentiating effect of thymol at heterologously expressed vertebrate GABA<sub>A</sub> receptors with different subunit combinations**

Thymol, 1  $\mu$ M-100  $\mu$ M, potentiated the EC<sub>20</sub> GABA response mediated by human ionotropic GABA receptors with the subunit combinations  $\alpha 1\beta 1\gamma 2s$ ;  $\alpha 6\beta 3\gamma 2s$ ;  $\alpha 1\beta 3\gamma 2s$ ; there was no difference between subunit combinations with respect to the degree of potentiation induced by a given concentration of thymol within this range. Data points are shown as the mean of 3-6 observations  $\pm$  one s.e.m.



**Figure 4.6 Flumazenil does not affect the GABA-potentiating activity of thymol at heterologously-expressed human  $\alpha 1\beta 3\gamma 2$ s GABA<sub>A</sub> receptors**

(a) Enhancement of EC<sub>20</sub> GABA by co-application of thymol and flumazenil. This increase in GABA receptor activity was not significantly different from that elicited by thymol alone (b).  
 (c) Data from 4 oocytes, shown  $\pm$  one s.e.m.

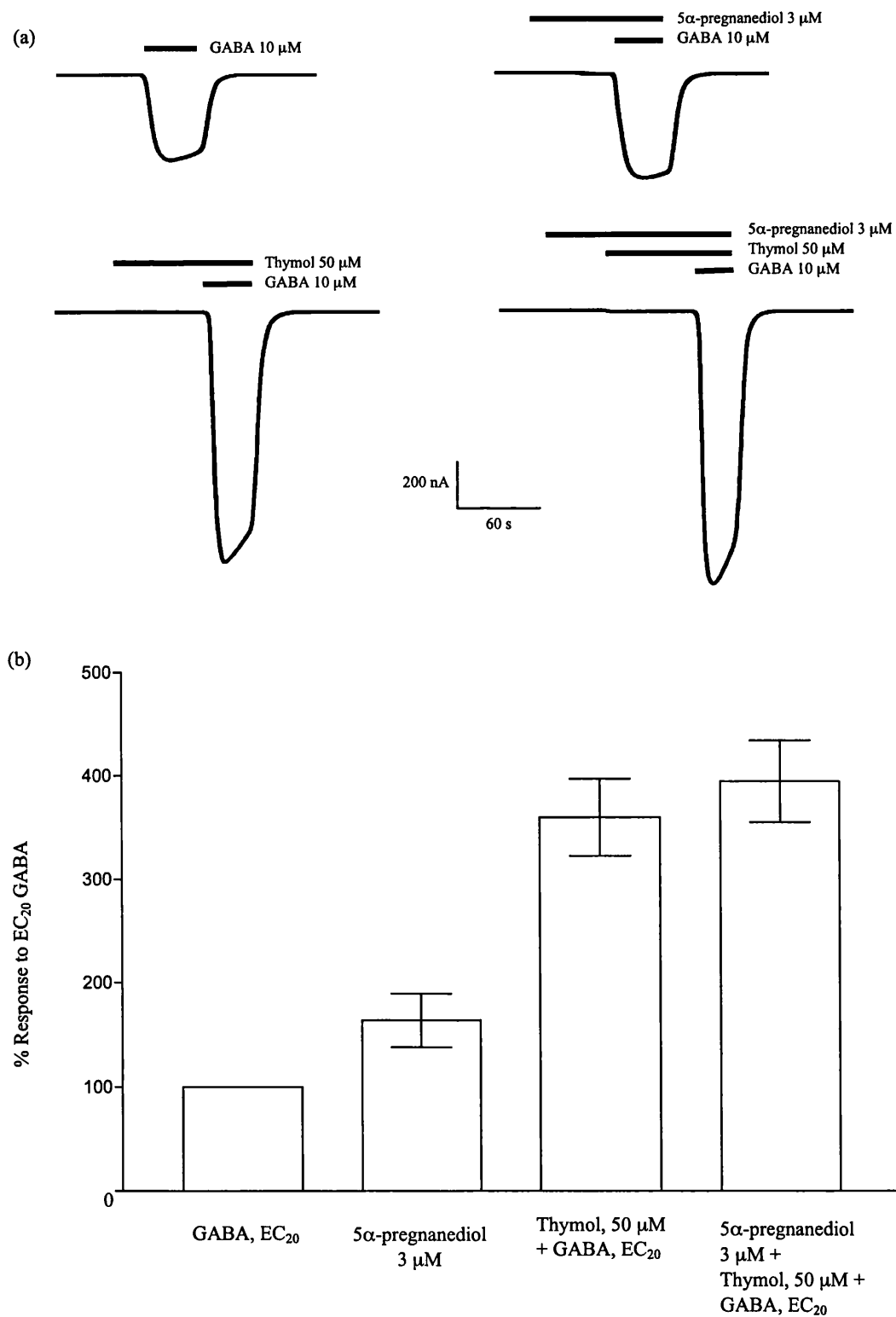


**Figure 4.7 The co-application of 3-hydroxymethyl- $\beta$ -carboline (3-HMC) with thymol does not affect thymol enhancement of the EC<sub>20</sub> GABA response in *Xenopus* oocytes expressing human  $\alpha 1\beta 3\gamma 2s$  GABA receptors**

(a) Sequential recordings from a single oocyte.

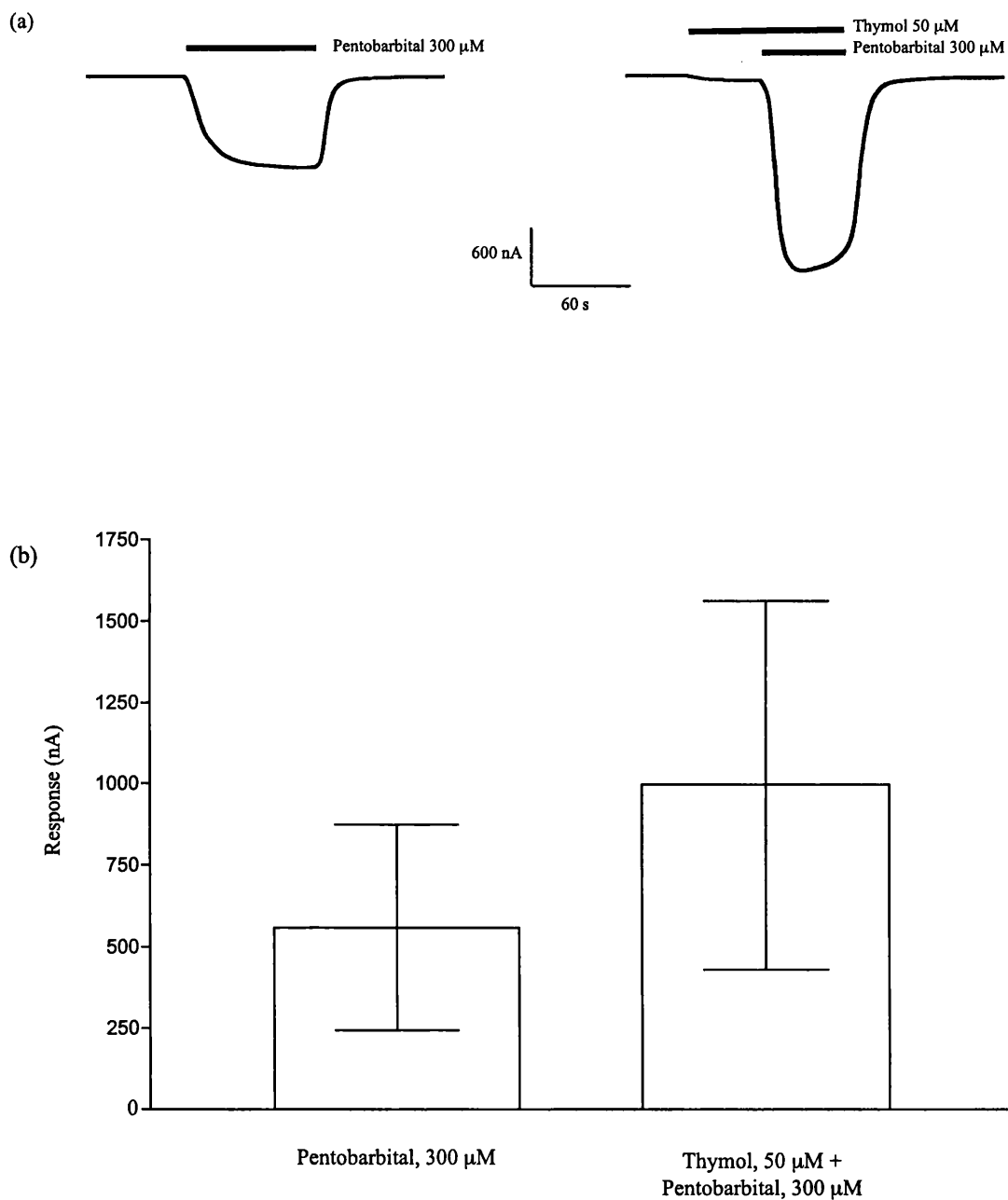
(b) Collated data from several oocytes showing the mean of 3 results,  $\pm$  one s.e.m.





**Figure 4.8 The steroid 5α-pregnane-3α, 20α-diol (5α-pregnanediol) has no significant effect on the thymol enhancement of the human  $\alpha 1\beta 3\gamma 2s$  GABA receptor-mediated response to GABA**

(a) Traces recorded sequentially from a single oocyte.  
 (b) Mean data ( $n = 4$ ) shown  $\pm$  one s.e.m.



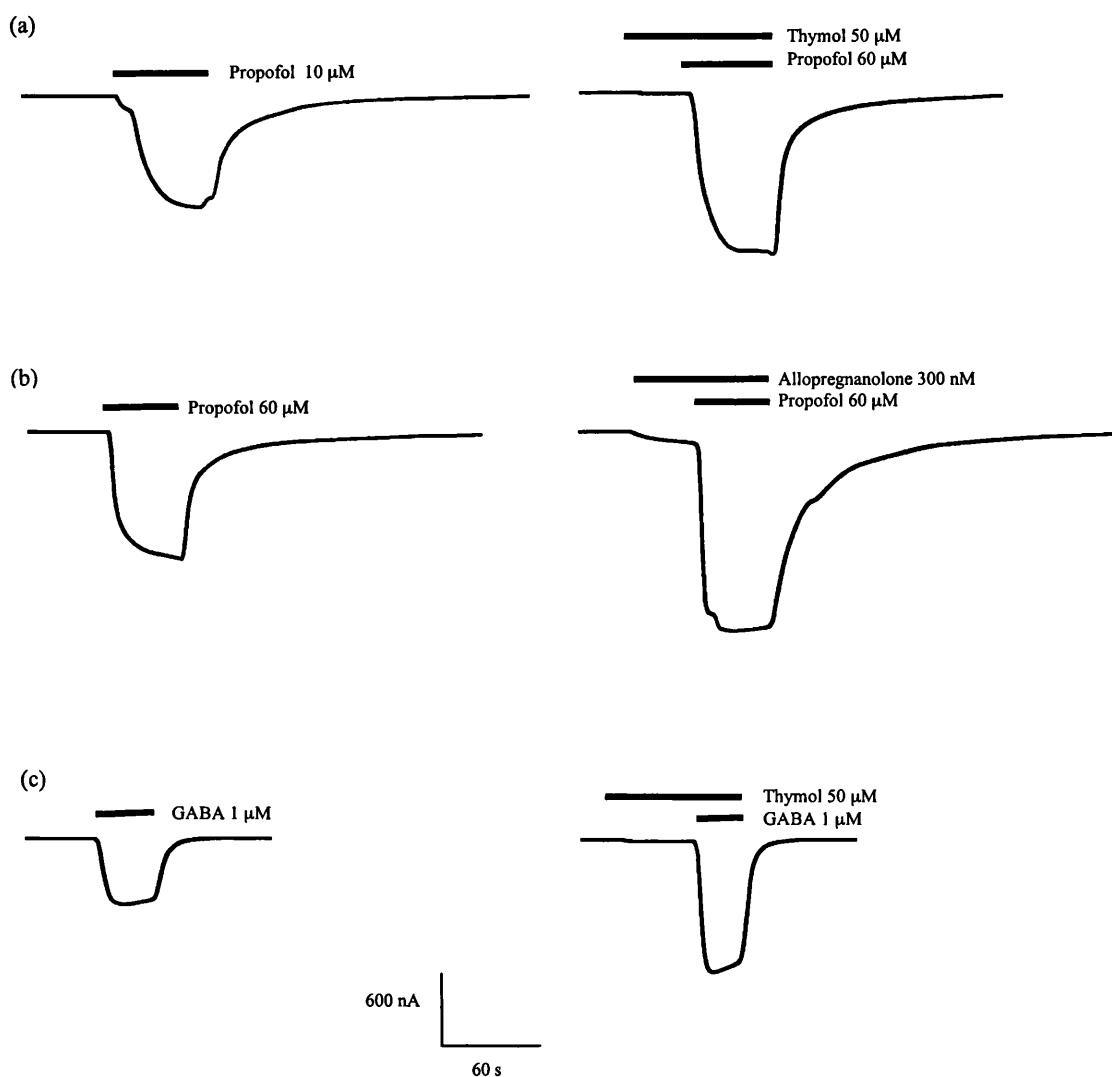
**Figure 4.9 Potentiation of the human  $\alpha 1\beta 3\gamma 2$ s GABA<sub>A</sub> receptor response to 300  $\mu$ M pentobarbital by 50  $\mu$ M thymol**

Receptors were expressed in *Xenopus* oocytes and currents recorded with the voltage clamped at  $-60$  mV.

Thymol potentiates the response to pentobarbital at human  $\alpha 1\beta 3\gamma 2$ s GABA<sub>A</sub> receptor:

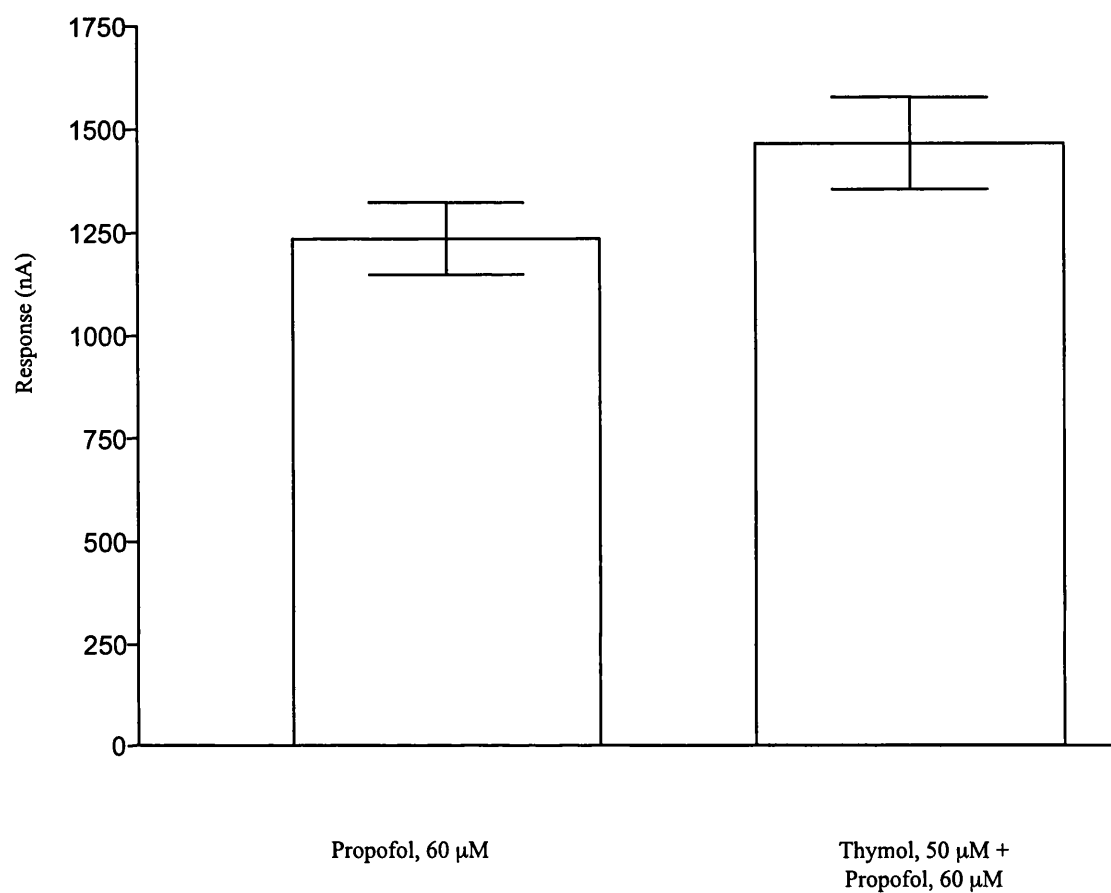
(a) Data from a single oocyte.

(b) Mean data from 4 experiments, shown  $\pm$  one s.e.m.



**Figure 4.10 Thymol-induced potentiation of the human  $\alpha 1\beta 3\gamma 2s$  GABA<sub>A</sub> receptor response to propofol**

(a) The current amplitude elicited by propofol showed a small (for thymol) but significant increment in the presence of thymol. For comparison, (b) potentiation of the propofol response by allopregnanolone and (c) the response to EC<sub>30</sub> GABA, which is of the same relative amplitude as that of 60  $\mu$ M propofol, and its enhancement by thymol are also shown. All recordings are from the same cell.



**Figure 4.11 Enhancement of the response to 60  $\mu$ M propofol by 50  $\mu$ M thymol at a human GABA<sub>A</sub> receptor**

Human  $\alpha 1\beta 3\gamma 2s$  GABA<sub>A</sub> receptors were expressed in *Xenopus* oocytes and the oocyte membranes voltage-clamped at  $-60$  mV. Data points represent the mean response from 4 oocytes, shown  $\pm$  one s.e.m

## **4.4 Monoterpenoid interaction with recombinant mammalian GABA receptors**

### **4.4.1 *Thymol is a positive modulator of human GABA<sub>A</sub> receptors and also has direct agonist activity***

In Chapter 3, the potentiating effects of the phenolics thymol, eugenol and carvacrol on a heterologously expressed insect GABAR were demonstrated. The results presented here show that thymol also potentiates the actions of GABA at a recombinant human GABA<sub>A</sub> receptor. Modulation of the human  $\alpha 1\beta 3\gamma 2$ s receptor was dose-dependent over a similar concentration range as that seen for potentiation of RDL<sub>ac</sub> homomers, although it appears that thymol is less potent on the mammalian than the insect model; a given concentration of thymol produced a lower potentiation at the GABA<sub>A</sub> receptor than at the insect receptor (See Figures 3.8 and 4.2). A small but definite effect of thymol applied alone was seen at both mammalian and insect recombinant receptors when high concentrations were tested. This was likely to have been the result of direct agonist activity of thymol at GABA<sub>A</sub> receptors, as uninjected eggs did not produce responses to thymol at equivalent concentrations; furthermore, direct agonist activity of thymol has recently been described at rat  $\alpha 1\beta 2\gamma 2$  receptors expressed in HEK cells (the potentiatory effect was not investigated) [176]. In the invertebrate receptor assay, potentiating effects of concentrations of thymol above 100  $\mu$ M thymol could not be tested as they caused instability of membrane currents in expressing cells, whereas in this, vertebrate equivalent the solubility of thymol and the decrease in potentiation levels at concentrations above 100  $\mu$ M were the limiting factors.

Given that thymol is able to potentiate GABA activity at the  $\alpha 1\beta 3\gamma 2$ s GABARs as well as at RDL<sub>ac</sub> homomers, it could be postulated that other volatile phytochemicals, especially eugenol and carvacrol, may also have this activity. Recently, it was found that eugenol, citronellol, pinene and citronellal all potentiated GABA responses in *Xenopus* oocytes injected with whole rat brain mRNA [285]. Although the subunit composition of the receptors formed was not determined, the findings of the aforementioned study suggest that the potentiation of GABA action at vertebrate ionotropic GABA receptors, by plant derived monoterpenoids and other similar compounds, is not restricted to those structures with a phenolic functional group.

Structure-activity studies of monoterpenoids on both insect and vertebrate GABA receptors may reveal that some are actually highly specific for insect receptors, thereby indicating potentially selective agents for development as insecticides. In this study, instead of examining the structure-activity relationships, the binding site and molecular requirements for the interaction of monoterpenoids with GABARs were examined; the variety of pharmacological and molecular tools available for the GABA<sub>A</sub>R made its use preferable over RDL<sub>ac</sub> homomers. Binding site information is important in the study of insecticidal activity, as it allows the researcher to determine if the site of action is shared by any other previously known ligands of the receptor. By grouping ligands in such a way, the potential for cross-resistance within and between groups can be studied. Furthermore, even if some compounds in an identified binding site class are not useful clinically or agriculturally, they could still be used as tools to study molecular interactions at that site, via competition or radio-ligand binding studies for evaluation of related structures. Knowledge of both the optimal pharmacophore and the nature and shape of the binding site allows the drug-receptor interaction to be modelled and this is important in rational drug design.

#### **4.4.2 Effect of thymol on the GABA dose-response curve**

The significant leftwards shift of the GABA dose-response curve obtained from  $\alpha 1\beta 3\gamma 2$ s-expressing oocytes demonstrates that thymol can increase the efficacy of submaximal concentrations of GABA, but does not greatly affect the maximum response. This suggests that thymol is specifically facilitating the manner in which GABA binds to or activates the receptor, but cannot increase the current flow above the maximum possible achieved by GABA alone. The Hill coefficient of the GABA dose-response curve was also decreased in the presence of thymol, suggesting a reduction in the number of GABA molecules required to activate the channel; unliganded GABARs require two or more GABA molecules for channel activation, whereas the thymol-modulated receptor may only require one. In these respects, the action of thymol on GABA<sub>A</sub> receptors is similar to that of the insecticide  $\delta$ -HCH on RDL<sub>ac</sub> homomers but not to that of  $\delta$ -HCH on GABA<sub>A</sub>Rs; on RDL<sub>ac</sub> homomers,  $\delta$ -HCH shifts the dose-response curve to the left, decreasing nH without affecting the maximal response [116]; on GABA<sub>A</sub>Rs, however,  $\delta$ -HCH shifts the dose-response curve to the left but also depresses the amplitude of the maximal response [272].

#### **4.4.3 Effect of changing subunit composition on thymol action**

Changing the subunit combination of the receptor is useful as a first step towards elucidating modulator binding sites, as it is a relatively quick method of identifying portions of the protein, in the form of specific subunits or subunit interfaces, important for mediating the effects of a ligand. Neither changing the  $\alpha 1$  subunit to  $\alpha 6$  nor changing the  $\beta 3$  subunit to  $\beta 1$  had any effects on the potency or maximal potentiation of thymol on GABA<sub>A</sub> receptors. Substituting  $\alpha 1$  to  $\alpha 6$  renders receptors otherwise sensitive to the majority of benzodiazepines unresponsive to the majority of this class of compound, with the exception of certain benzodiazepine site ligands, such as the partial inverse agonist Ro 15-4513 [265]. This substitution was used previously in an attempt to characterise a novel positive allosteric modulator of GABA<sub>A</sub> receptors: the activity of (+)-ROD188 was compared on benzodiazepine-sensitive (rat  $\alpha 1\beta 2\gamma 2$ ) and benzodiazepine-insensitive combinations of subunits (rat  $\alpha 1\beta 2$ , rat  $\alpha 6\beta 2\gamma 2$ ). In fact, this compound was most active at receptors containing the  $\alpha 6$  isoform in  $\alpha x\beta 2\gamma 2$ , where  $x = 1, 2, 3, 5, 6$ . Thymol had no particular selectivity for  $\alpha 1$  or  $\alpha 6$ , suggesting that it does not act via the benzodiazepine binding site, nor the (+)-ROD188 site [248].

Loreclezole potentiates the actions of GABA at both RDL<sub>ac</sub> and GABA<sub>A</sub> receptors, the determinants of potency at the loreclezole site on GABA<sub>A</sub> receptors being shared by the positive modulatory actions of etomidate and DMCM [107,242]. As there are no competitive inhibitors of loreclezole available, the most readily available way in which to test if a compound shares a site on the receptor with loreclezole is to investigate if it, like loreclezole, has reduced potency on  $\beta 1$ -containing receptors in comparison to  $\beta 3$ -containing receptors; the activity of thymol was not affected by this change in recombinant  $\alpha 1\beta x\gamma 2$ s GABA<sub>A</sub>Rs (where  $x = 1$  or  $3$ ). This study shows that, unlike loreclezole, thymol potentiates the GABA response at  $\alpha 1\beta 1\gamma 2$  or  $\alpha 1\beta 3\gamma 2$  receptors with no difference in EC<sub>50</sub> or efficacy, suggesting that thymol does not interact via the loreclezole binding site.

#### **4.4.4 Benzodiazepine and $\beta$ -carboline binding sites**

Further evidence that thymol does not interact with the benzodiazepine binding site was provided by competition studies using the ligands flumazenil and 3-HMC. Certain benzodiazepines, such as the allosteric enhancer flunitrazepam, are active on many native

insect receptors and also GABA<sub>A</sub> receptors but do not have activity at RDL homomers [116,219]. It has been suggested that the activity of these benzodiazepines on insect receptors depends on the presence of more than one subunit type, as it does in vertebrates [119]. Other benzodiazepines, such as 4'-chlorodiazepam, do potentiate agonist activity at RDL homomers [116]. However, it is relatively difficult to perform competition studies between two compounds that both potentiate GABA responses, and antagonists are preferred for this purpose: for this reason, the known potent GABA<sub>A</sub> receptor antagonist flumazenil and inverse agonist 3-HMC were chosen for study.

Flumazenil (Ro15-1788), at 1  $\mu$ M, was used in a previous functional study to investigate the interaction of the positively modulating  $\beta$ -carboline ZK 91085 with the rat recombinant  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor: potentiation by 0.1 mM and 0.7 mM ZK 91085 was reduced by 70 % and 51 % respectively in the presence of flumazenil [116], indicating an action at the benzodiazepine binding site. However, 1  $\mu$ M flumazenil had no effect on the activity of (+)-ROD188, which therefore does not compete with benzodiazepine binding [248].

Not all  $\beta$ -carbolines interact exclusively via the benzodiazepine/ $\beta$ -carboline binding site, for example, one of the best characterised  $\beta$ -carbolines, DMCM, acts as an inverse agonist via this site but, at high concentrations, positively modulates via the loreclezole site. So far, there is no evidence that 3-HMC displays the latter property at GABA receptors and therefore is more useful in competition studies; it blocks GABA action at the cockroach D<sub>f</sub> motor neuron, and, although it does not have inverse agonist activity at RDL homomers, it was reported to competitively antagonise the potentiating effects of 4'-chlorodiazepam at this receptor [116].

Both flumazenil and 3-HMC have high binding affinities to GABA<sub>A</sub> receptors. Therefore, assuming that the affinity of thymol is not particularly high, a substantial reduction in thymol activity associated with its displacement from the receptor would be expected if thymol acted via the benzodiazepine/ $\beta$ -carboline binding site. In this case, neither compound had a significant effect on the activity of thymol, suggesting that there is no competition for binding site between thymol and compounds of these classes.



#### **4.4.5 Interaction with the steroid binding site**

Functional competition studies where both agents potentiate the effects of the agonist are the most difficult to interpret; it is not possible to predict exactly how the potentiating activities of two different compounds, whether acting at the same binding site or not, will be integrated by the receptor in terms of overall conformational changes and subsequent effect on the response. Previous literature [248] used the rationale that an interaction between compounds would manifest as a potentiation by the dual combination less than the (theoretical) additive effect of the two compounds applied separately. The fact that 5 $\alpha$ -pregnanediol potentiation of the GABA response was much lower than that of thymol suggests that a functional interaction might result in a combined enhancement lower even than that produced by thymol alone.

When applied together the combined enhancement by thymol and propofol was actually higher than that produced by thymol alone. An unpaired t-test revealed that there was no significant difference between the expected combined potentiatory actions of thymol and 5 $\alpha$ -pregnanediol, calculated by summation of the individual percentage potentiations, and the actual potentiation, allowing the conclusion that the effects of thymol and 5 $\alpha$ -pregnanediol are entirely additive. This result suggests that thymol does not act via the steroid binding site.

#### **4.4.6 Interaction with a barbiturate site of action**

Barbiturates potentiate the effects of GABA at GABA<sub>A</sub> receptors, and also have a direct agonist effect [199]. As competition studies between two potentiating compounds can be liable to ambiguities in their outcome, it could prove more conclusive to study the ability of thymol to potentiate the agonist effect of pentobarbital; if thymol can potentiate this activity it is possible that thymol binds to a site allosteric to the barbiturate agonist binding site; if thymol cannot enhance barbiturate agonist action, or shows only marginal potentiation, it is likely that thymol binds via the same site. The pentobarbital response was potentiated significantly by thymol (see Figure 4.9), suggesting a distinct mode of action. Response amplitudes to pentobarbital as an agonist are often highly variable, consistent with the results obtained in this study. For example, in the study investigating (+)-ROD188 action at the pentobarbital agonist site, the mean potentiated pentobarbital current was

given with an s.e.m of  $\pm 37\%$ , responses to other drugs in this paper typically had s.e.m.s of between  $\pm 5$  and  $\pm 12\%$  (from 7 examples chosen at random). Furthermore, although this paper claims that (+)-ROD188 potentiates the pentobarbital current, it does not state if the potentiation was significant or not [248].

#### **4.4.7 Interaction with propofol**

Thymol-induced potentiation of the direct agonist effect of propofol, which also acts as a positive allosteric modulator at GABARs, was also examined. Although the amplitude of the propofol current was found to be significantly greater in the presence of thymol, the degree of propofol potentiation by thymol appeared rather low in comparison to thymol-induced potentiation of  $EC_{20}$  GABA responses. Preliminary tests were then carried out on a single oocyte to see if it could be determined whether the thymol-potentiated propofol current was in fact reduced, on account of an interaction between the compounds: 5 $\alpha$ -pregnan-3 $\alpha$ -ol-20-one (allopregnanolone) was used to assess the degree by which the propofol response could be potentiated by another positive modulator; furthermore, to compare potentiation of a functionally equivalent dose of GABA, thymol enhancement of  $EC_{30}$  GABA (equivalent to 60  $\mu$ M propofol in terms of the fraction of the maximal GABA response produced) was assessed. It was found that the thymol potentiation of the propofol response, the allopregnanolone potentiation of the propofol response and the thymol potentiation of the  $EC_{30}$  GABA response were all similar in terms of the percentage increase in current above control, this provides further evidence that potentiation of the direct effect of propofol by thymol is not inhibited by competition with propofol, and therefore thymol does not act via the propofol agonist site.

#### **4.4.8 The site of action of thymol on GABA receptors, as determined by functional competition studies**

The evidence reported here suggests that thymol does not share sites of action with many of the most widely investigated allosteric modulators of GABA<sub>A</sub> activity, these being benzodiazepines,  $\beta$ -carbolines, barbiturates, propofol, loreclezole and steroids. It is still contentious as to whether the barbiturates and propofol act as agonists and positive modulators via the same or distinct sites: a range of point mutations on GABAR subunits influence both the agonist and modulator activities of pentobarbitone and/or propofol

[9,200] leading some authors to conclude that the sites for both activities are identical, however, such mutations could possibly be affecting aspects of transduction, rather than binding [25]. The simplest conclusion is that thymol has a mode of action different from that of pentobarbital and propofol as GABAR agonists, and possibly also as modulators.

It should be considered that, without binding data, there is a chance that an inhibition by one ligand on the potentiatory activity of another may be the result of an allosteric action, and not competition. Furthermore, differences in activity at different subunits may reflect differential transduction rather than differential binding. In this study, the lack of positive results in all experiments suggests that neither the binding site nor transduction domains were affected in any of the test conditions.

To assess sites of interaction on the receptor, binding studies have historically been more commonly employed as a first line method of studying ligand-receptor interactions than functional competition studies. For reasons outlined in the previous paragraph and below, binding data make a useful accompaniment to functional studies. Obtaining data on binding kinetics prior to functional studies can predict the appropriate concentrations of competing agents to use in functional studies. To ensure sufficient displacement of the compound of interest occurs to see an effect on the response, certain types of compounds are not amenable to binding assays; for example,  $\alpha$ -endosulphan, a GABAR antagonist, readily partitions into membranes and this precludes determination of affinity [72]. Monoterpenoids are also known to partition into membranes and therefore binding studies may be similarly problematic. However, a recent study has successfully dealt with differential displacement of the radioligands [ $^3$ H]muscimol and [ $^3$ H]MK801 (NMDA receptor antagonist) from mouse cortical membranes by linalool [37], and  $\alpha$ -thujone was shown to displace [ $^3$ H]EBOB binding [110]. Perhaps further studies should therefore attempt to confirm that the reason for the lack of competitive effects between thymol and other GABAergic agents is not because the affinity of thymol for its site is too great to allow even partial displacement by the other ligands to occur.

#### **4.4.9 Further possible mechanisms of thymol action**

In this study, thymol has tentatively been termed a positive allosteric modulator of GABA receptors, however, as yet no putative allosteric site for thymol has been identified. One review of allosteric receptors describes allosteric modulation as the interaction of a molecule with “sites of action on receptors or carrier molecules that are different and distant from signal substance or substrate binding sites” [100]. Another, more inclusive definition states that an allosteric interaction between receptor ligands occurs, not by “classical mutual exclusion,” but via “specific,” “topographically and stereochemically distinct sites” [64]. It is therefore interesting to note that (+)-ROD188 was described in a recent publication as a positive allosteric modulator of the GABAR, simply by the virtue that it stimulated GABA induced currents at GABARs in a concentration dependent fashion and induced negligible currents by itself (amplitude not given), despite the fact that no high affinity binding sites were demonstrated; a weak interaction with the benzodiazepine site was found but was clearly not the site through which potentiation was mediated. Slight selectivity was shown for  $\alpha 6$  containing receptors, but this does not necessarily reflect binding preferences [248]. To confirm the interaction of compounds, such as (+)-ROD188 or thymol, with GABARs as allosteric, first a “specific” site for interaction on the receptor should be described. Otherwise, there may be the possibility that there is an intermediary site of action; for example, thymol could be activating cellular components of oocytes that interact with GABA receptors. Alternatively, thymol could be affecting properties of the lipid bilayer, thereby indirectly altering the conformation of the multimeric receptor.

### ***Does thymol affect GABA<sub>A</sub>Rs by interaction with the lipid bilayer?***

The previous study addressing thymol action at heterologously expressed GABA<sub>A</sub>Rs [176] compared its activity with propofol, an intravenous anaesthetic compound, however, perhaps a comparison between thymol and volatile anaesthetic agents would also be useful. Many compounds which have similar properties to thymol, being volatile and lipophilic in nature, share common anaesthetic effects in humans [136]. Anaesthetics in fact encompass a wide variety of non-volatile, volatile and gaseous substances including trichloroethanol, barbiturates, steroids, xenon, propofol and isoflurane [9]. Anaesthetics generally have low affinity for multiple binding sites, and have been reported to affect many aspects of cell function, therefore possessing “non-specific” modes of action (although it should be noted that steroids are perhaps an exception, being particularly potent and selective for GABA<sub>A</sub>Rs at concentrations relevant to anaesthesia). However, these compounds generally affect ion channels at therapeutic concentrations, and other cellular processes at levels higher than those producing anaesthesia [170]. For many years anaesthetics were believed to act by disordering the lipid bilayer, and recently certain observations have added weight to this argument, for example, detergents and free fatty acids can affect receptor channel function by changing bilayer properties [137], and theories of how this disordering effect might affect the gating of intrinsic ion channels have been put forward [136].

Monoterpenoids have been studied for their membrane disordering properties in mitochondria with positive results, the most potent, and also the most lipophilic of these was methyl-limonate, which uncoupled 50 % of oxidative phosphorylation at 200  $\mu$ M and inhibited 50 % of inner membrane electron transfer at 1 mM [18]. These concentrations are at the upper end of the concentration range of the terpenoids tested on oocytes in Chapter 3, and mitochondrial uncoupling or disruption of PM integrity could provide the reason why carvacrol was particularly liable to induce rapid loss of membrane potential in oocytes at concentrations higher than 100  $\mu$ M. Thymol, eugenol and carvacrol did not affect lipid bilayer integrity at the concentrations required for potentiation, as the leak currents in uninjected oocytes were negligible (less than 10 nA for 100  $\mu$ M thymol and 1 mM eugenol) when these compounds were applied alone, confirming that the plasma membrane was intact. Furthermore, if the oocyte mitochondria had been affected at these concentrations, depolarisation of the plasma membrane would have occurred when thymol, eugenol or carvacrol were applied alone to uninjected oocytes, as the membrane potential of a cell is highly dependent on the supply of ATP from mitochondrial activity to power active ion transport across the membrane.

Actions of monoterpenoids as membrane-perforating and membrane-stabilising agents have been documented; for example, carvacrol enhances the transmembrane penetration of other drugs [142]. However, regarding the effect of membrane disordering on receptors, it is contentious as to whether the degree of fluidisation caused by general anaesthetics (at relevant concentrations) is significant or not [136,170]. Considering the mounting evidence for the action of many of these compounds at discrete sites on the ligand-gated ion channels themselves, especially in the case of GABA<sub>A</sub> receptors, the possibility that they affect channel gating indirectly, via the plasma membrane, now seems unlikely. For example, gaseous anaesthetics are active at a distinct set of ion channels from volatile anaesthetics [275,276], and it is unlikely that perturbations of the lipid bilayer differentially affect different channels.

### ***Intracellular constituents***

The widespread actions of anaesthetics led to the question of whether they were acting on a unifying intracellular component. This was disproven in the case of GABA<sub>A</sub>Rs, as potentiation of GABA<sub>A</sub>R activity by anaesthetics also occurs in membranes separated from all intracellular constituents [151]. Nevertheless, it is interesting that both thymol and eugenol appear to affect processes which influence intracellular Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>), as Ca<sup>2+</sup> controls a range of cellular activities, such as secretion, neurotransmitter release, muscle contraction and Ca<sup>2+</sup> sensitive enzyme activity; enzymes regulated by Ca<sup>2+</sup> include kinases and phosphatases that affect ligand-gated ion channel activity [204]. The Ca<sup>2+</sup> concentration gradients across the cell membrane and the membranes of internal stores are steep, and therefore the activation of processes which lead to Ca<sup>2+</sup> entry or release have profound effects on [Ca<sup>2+</sup>]<sub>i</sub>. Even if [Ca<sup>2+</sup>]<sub>i</sub> or Ca<sup>2+</sup>-regulated processes do not underlie thymol/eugenol induced potentiation of GABARs, it could be that the effects of thymol and eugenol on [Ca<sup>2+</sup>]<sub>i</sub> contribute to the physiological activities observed in response to these terpenoids.

Thymol is potent at releasing Ca<sup>2+</sup> from internal stores in both nerve and muscle [138,140,195] and, interestingly, calcium release from the sarcoplasmic reticulum (SR) is a property shared by the anaesthetics chloroform and halothane [204]. However, the actual consequences of thymol-induced calcium release appears to vary between preparations: thymol is reported to be one of the most potent stimulators of Ca<sup>2+</sup> induced Ca<sup>2+</sup> release from SR, which leads to raised cellular [Ca<sup>2+</sup>]<sub>i</sub> and, consequentially, muscular contraction [109]. However, in retinal rod photoreceptors, thymol-induced increases in [Ca<sup>2+</sup>]<sub>i</sub> were only transient, leading to speculation that, in this case, Ca<sup>2+</sup> release activated a Ca<sup>2+</sup> transporter which then lowered the [Ca<sup>2+</sup>]<sub>i</sub> to below the original level [140]. Release of Ca<sup>2+</sup> from intracellular stores also occurs in invertebrates, as demonstrated by monitoring Fura-2 fluorescence in snail (*H. pomatia*) neurones [138].

Although there is some evidence that eugenol may release Ca<sup>2+</sup> from intracellular stores [174], this probably only occurs at high concentrations (3-12 mM), which induce contractures of toad skeletal muscle; lower concentrations (0.1-2.5 mM) of eugenol blocked K<sup>+</sup> - induced contracture [146]. This effect of eugenol at low concentrations may have been due to eugenol blockade of Ca<sup>2+</sup> channels, and this was also the likely reason for the negative inotropic effect of eugenol in guinea pig heart muscle [227]. In some nerve preparations, such as *H. pomatia* neurones, eugenol also blocks Ca<sup>2+</sup> currents. However,

eugenol probably has different effects on distinct mechanisms of calcium entry and exit from the cell, as in rat dorsal root ganglion (DRG) neurons, 1 mM eugenol activated a  $\text{Ca}^{2+}$  current [188] and eugenol-mediated vasodilatation of rabbit aorta appeared to be via inhibition of both uptake and extrusion mechanisms for  $\text{Ca}^{2+}$  [185].

Although the information regarding the effects of thymol and eugenol on  $\text{Ca}^{2+}$  currents and  $[\text{Ca}^{2+}]_i$  regulation in nerve and muscle cells is still rather sketchy, the possibility that thymol, eugenol and other monoterpenoids may be raising oocyte internal  $\text{Ca}^{2+}$  levels, which then activates a  $\text{Ca}^{2+}$ -dependent, GABAR-modulating mechanism should not be ruled out at this stage. Another interesting aspect of this research is that, so far, it appears that the primary  $\text{Ca}^{2+}$ -related effect of thymol is intracellular  $\text{Ca}^{2+}$ -store release, whereas eugenol clearly affects  $\text{Ca}^{2+}$  channels and/or uptake mechanisms, and this could underlie the differential utility of these compounds as anaesthetics. A tentative hypothesis would be that eugenol relaxes muscles directly via blockade of calcium channels; and that central nervous system depression could be effectively achieved via a combination of reduced excitatory neurotransmitter release from nerve terminals, due to reduction of  $\text{Ca}^{2+}$  entry and subsequent fusion of vesicles containing neurotransmitter to the presynaptic membranes [204], the concomitant decrease in GABA release could be compensated for by potentiation of GABA activity at the receptor level. However, it is not fully known how the anaesthetic state is achieved by any particular compound, so this model is entirely speculative.

#### ***Single or multiple monoterpene binding sites on GABARs?***

Aside from affecting the lipid bilayer or intracellular components, there are other putative explanations for the reason why many volatile, lipophilic compounds act on so many regulatory proteins [25], for example, it has been suggested that small volatile compounds do not have a binding site per se (consistent with the lock and key theory ligand-receptor interaction) but interact opportunistically with multiple sites on receptors [136]. Yet the plausibility of this theory is reduced in light of the findings that two specific point mutations confer ethanol and enflurane sensitivity to GABA  $\rho 1$  receptors [169]. Even if this mutation was in a transduction domain rather than a binding domain it seems unlikely that multiple small binding sites across the receptor would converge on the same two amino acids, especially as propofol sensitivity was not conferred. Furthermore, residues in the N-terminal domain influence ethanol inhibition of nAChRs [170], providing evidence that ethanol binds to a specific, extracellular area on these receptors; therefore ethanol



action is probably not via the lipid bilayer nor activation of an intracellular component.

Often, the volatile anaesthetics are referred to as a group in terms of GABA potentiatory activity. However, it appears that not all volatile anaesthetics depend on the same portions of the protein for activity [96]. Furthermore it appears that volatile convulsants and volatile anaesthetics share different sites of action on GABARs [127]. This leads us to suspect that there also may not be a single “monoterpenoid binding site” on GABARs but perhaps different sites for different structures: there is evidence for  $\alpha$ -thujone binding to the convulsant site [110] but, if thymol were to share this property, it would be unusual for a positive allosteric modulator. Evidence that at least some monoterpenoid binding sites may be shared by several structures comes from the observation that [3H]-menthol binding to guinea-pig lung is displaced by eugenol and camphor (and also capsaicin) [274]. There may be multiple sites mediating the effect of one structure in the manner proposed for many anaesthetics, where sites mediating potentiatory activities are probably separate from those through which a direct agonist effect occurs [176]; dual potentiatory activity and (less potent) agonist activity is another characteristic of thymol that is shared by anaesthetics.

The putative thymol binding site on GABA<sub>A</sub>Rs could be investigated further by observing the activities of other monoterpenoids at these receptors: if another monoterpenoid could be found which competitively inhibited the potentiation of thymol, but had no activity itself, analogous to the activity of flumazenil with respect to other benzodiazepines, this may provide additional evidence that binding was to specific sites and not just “opportunistically” at random hydrophobic pockets. Even if a specific binding site on GABA receptors is discovered, this does not preclude the discovery of monoterpenoid action at other neuronal receptors; there are many other GABAergic compounds with multiple targets: PTX blocks several ion channels, such as vertebrate glycine-gated-channels [155], invertebrate L-glutamate-gated chloride channels (L-GluRs) [210], and acts at some nAChRs [169]; BIDN and fipronil also block L-GluRs [210,282]; general anaesthetics affect the functions of many transmitter-gated ion channels such as excitatory ionotropic glutamate, nicotinic acetylcholine and 5-HT<sub>3</sub> receptors, inhibitory strychnine-sensitive glycine receptors and GABA<sub>A</sub>Rs, modulation can be positive or negative or absent depending on the agent and the receptor [112]. Volatile anaesthetics induce neuronal inhibition via blockade of excitatory pathways as well as via stimulation of inhibitory ones [92]. Propofol also acts to suppress L-type Ca<sup>2+</sup> channels in the heart and sensory motor neurones of the spinal cord [99]; Ca<sup>2+</sup> channel blockade, especially in heart muscle, appears to be a property shared by eugenol. The variety of different target sites or mechanisms of

action of anaesthetics are thought to underlie the different elements of the anaesthetic state [90]. Thymol and eugenol also have multiple bioactivities but clearly further work is required to establish the precise targets behind the majority of these effects.

Thymol appears not to act as an anaesthetic agent in contrast to eugenol and although this could be due to their relative activities at other targets, there is a possibility that differential effects at GABA<sub>A</sub>Rs might be the cause. Future studies could perhaps compare the potentiatory and direct effects of thymol and eugenol at various GABA<sub>A</sub>R subunit combinations to test this theory.

#### ***4.4.10 Implications with respect to use of thymol and related compounds as insecticides***

The likelihood that the results of the experiments carried out in this Chapter are also applicable to the interaction of thymol with insect GABA receptors is high, although of course this requires confirmation. Despite the fact that allosteric modulator and convulsant antagonist binding sites differ between GABA<sub>A</sub> and insect receptors in terms of binding affinities and pharmacology at each individual site, the spectrum of compounds which occupy the distinct, discrete binding sites, is the same for both type of receptor. As thymol has similar activities at insect and human GABA<sub>A</sub> type GABARs, it is likely to occupy equivalent binding sites on the two receptor types and therefore probably would be shown not to interact with insect GABAR benzodiazepine, loreclezole, steroid, barbiturate or propofol binding sites in equivalent studies.

If thymol, carvacrol and eugenol do represent examples from a group of insecticidal compounds that have a novel mode of action on GABA receptors, as these results suggest, they could represent a new avenue in pesticide, or even therapeutic, research; further investigations into the binding site and synthetic optimisation to improve GABAergic activity, pediculicidal activity, physicochemical or pharmacokinetic properties would probably yield chemicals with improved potential. Selectivity for the insect receptor may also be improved by chemical modification, however, data of GABAergic activity of other insecticides suggests that this is not necessarily a prerequisite for the success of the compound as an insecticide, as both lindane and dieldrin, for example, also act on mammalian GABA receptors [180]. These organochlorines have occasionally caused acute poisoning in mammals experiencing food shortage, as the breakdown of fatty tissue where

they accumulate leads to their sudden release into the body. However, the reason for their withdrawal as agricultural insecticides was not because of insufficient selectivity but due to their extreme stability and consequent environmental persistence [78].

### ***Summary and direction***

In addition to its actions at an insect GABA receptor, thymol has been shown to potentiate the agonist activity of GABA at heterologously expressed human GABA receptors. So far, it appears that thymol does not share a binding site with any of the following major classes of allosteric modulator: benzodiazepines,  $\beta$ -carbolines, barbiturates, propofol, steroids, loreclezole. Although this could mean that thymol binds to a completely different, and previously unexplored, GABA receptor site, the possibility that thymol may be acting on a unifying cellular component should not be entirely ruled out. Further experiments, perhaps utilising kinase inhibitors,  $\text{Ca}^{2+}$  imaging and different membranous expression systems may help to reject the latter hypothesis, whilst radio-ligand binding studies may lend support to the theory of a direct interaction at the receptor level.

Whether or not the efficacy of thymol in potentiating the action of GABA at heterologously expressed GABA receptors is, in any way, related to its insecticidal potency on human lice and their eggs remains to be confirmed.

## Conclusions and further development of this work

The incidence of head lice is increasing in the developed world, and is still highly prevalent in the undeveloped world. The main reason for the increase in frequency in the West is likely to be the continued propagation of insecticide resistant lice. Although physical intervention methods have been proposed, they are unlikely to be completely successful; as part of the adaptation to the parasitic state involves the ability to withstand all manner of grooming procedures, making the combing out of lice and eggs too time-consuming to be effectively implemented. Therefore unless new chemical treatments are introduced the problem is likely to continue escalating, and the normally innocuous condition of pediculosis capitis could, again, become a threat to health in this country.

The currently recommended chemical intervention methods are based on active ingredients from chemical classes with broad-spectrum insecticidal activity, and *in vitro* research suggests that, even in the absence of resistance, all have incomplete ovicidal activity. Although formulation can help, to ensure that all eggs are killed the recommended treatment regime is a 12 h application, repeated after 7 days in case any lice or eggs survived the first treatment. If head louse remedies were more ovicidal, the patient would not need to be exposed to the insecticide for such a length of time.

Essential oils and their constituent monoterpenoids have frequently been discussed for the purpose of head louse control, and several previous lines of evidence suggest their utility. However, a rational approach to *in vitro* work is necessary. For example, even if synergy between essential oil components is possible, there is little point screening random combinations of oils before the efficacy of individual oils is assessed. Similarly, it is important to assess the pediculicidal efficacy of different structural types of individual essential oil components, as their contribution to the activities of whole oils can then be determined in the future. The question of whether whole oils or constituents might provide a superior basis for a product could then be answered independently.

In order to minimise exposure to the patient and to the louse, thereby reducing the likelihood of adverse effects and resistance, respectively, the duration of insecticide application should be as short as possible. A treatment time of less than 3 h would provide convenience and reduce patient-insecticide contact time, it also would be important in the case of volatile oils, as the concentration on the scalp after application is likely to decrease

significantly with time, and this may increase the rate of resistance.

In this study, the relative short-term toxicities of various monoterpenoids and nerolidol, a sesquiterpenoid, were screened on lice and eggs. These results showed that the most effective pediculicidal monoterpenoids were monocyclic, and contained a single O-atom. Of the compounds selected, (+)-terpinen-4-ol had the highest activity, supporting the many incidences of anecdotal evidence for the efficacy of tea tree oil, which contains terpinen-4-ol as a major constituent, against head lice.

However, the ovicidal testing carried out so far suggests that although the ranking for activity on lice and eggs shows some similarities, for example, the higher potency of thymol over menthol and carvacrol, it also reveals important differences: nerolidol was more ovicidal than thymol, whereas it was ineffective in the pediculicidal study. Lice eggs are considered to be more difficult to kill than adults, and the fact that nerolidol appears to have specific ovicidal activity may indicate its potential for use in an anti-louse product. Although the recommended pediculicides currently contain only one active component, most likely due to licensing restrictions, it may be relatively easy to obtain a licence for a formulation with two distinct 'ovicidal' and 'pediculicidal' terpenoids, or whole essential oils, due to hallowed usage of the latter.

Future studies could investigate if the rapid killing times achieved *in vitro* might also be possible in the field by conducting clinical trials. Further *in vitro* work might also compare the efficacies of monoterpenoids with the currently available pesticides. In the case of personal parasite control, relative toxicity (insect vs. human) is more important than absolute insect toxicity.

Given the apparent selectivity of monoterpenoids towards certain insects, it is doubtful that a more suitable model could be found to screen for activity against head lice than the Orlando strain clothing louse. Although the house dust mite, in preliminary tests, appeared to show the same relative sensitivity to essential oils as the clothing louse, the same application methods cannot easily be applied to both species, as the dust mite is so small, and far more mobile; therefore, as the efficacy of an insecticide is notoriously not only dependent on the species but also the route of exposure, the house dust mite appears to be an unsuitable model. If the clothing louse is completely unavailable, other closely related lice of mammals, or larger mite species, could be considered instead.

The mechanisms of action of pediculicidal monoterpenoids should be investigated to determine the relative likelihood of toxic effects towards humans. Knowledge of the binding site and its structure-activity relationships would also facilitate the development of more selective or more effective agents. Previous reports suggesting a neurophysiological mode of action for monoterpenoids were supported by observational studies on lice and eggs in this thesis. To investigate this activity further, monoterpenoids were tested on an *in vitro* model of an insect GABA receptor, the *Drosophila* RDL<sub>ac</sub> homomer, expressed in *Xenopus* oocytes. Orthologues of this receptor have been found in a wide variety of insects and therefore also probably occur in lice, although this has not been explored so far. Confirmation of the existence of RDL in lice could be achieved by antibody staining of nervous tissue.

The homomeric RDL<sub>ac</sub> insect GABA receptor was identified as a novel target for monoterpenoids and eugenol, a structurally similar phenylpropanoid. Although preliminary observations suggested that the most effective pediculicidal monoterpenoid, terpinen-4-ol, was inactive at this receptor, thymol, eugenol and carvacrol all potentiated the actions of GABA by substantial degrees, and also showed agonist activity at higher concentrations. Potentiation of GABA receptor responses is a property shared by the insecticidal compound  $\delta$ -HCH, and modulation of neuronal inhibition may be a mechanism by which monoterpenoids cause either death, knock-down or both.

Thymol also potentiated responses to GABA at  $\alpha 1\beta 3\gamma 2s$  human GABA<sub>A</sub> receptors. Although this could mean that its use as an insecticide could result in cases of toxicity due to insufficient selectivity, activity at the same target site in insects and mammals does not preclude the success of an insecticide. The site of action of thymol on the  $\alpha 1\beta 3\gamma 2s$  receptor was investigated and, so far, functional studies have failed to find any interaction with benzodiazepine/ $\beta$ -carboline, barbiturate, propofol, steroid or loreclezole sites of action. Competitive binding studies between thymol and other radiolabelled GABA receptor ligands may yield further data to support these observations.

Furthermore, in light of the recently discovered activity of the monoterpenoid  $\alpha$ -thujone at the convulsant antagonist binding site on vertebrate GABARs, it would be interesting to investigate the effects of co-application of thymol with picrotoxin, BIDN or fipronil, or to observe if displacement of [<sup>3</sup>H]EBOB binding could be achieved with thymol. The activity of thymol at the A302S RDL<sub>ac</sub> homomeric receptor might also be examined. Although other positive allosteric modulators appear to act at sites distinct from the convulsant

binding site, the likelihood that thymol may bind there should be tested. The possibility of thymol acting indirectly via activation of an intracellular constituent could be addressed by electrophysiology on isolated membranes as described in [151].

Without an identified binding site or receptor mutant, it is not possible to convincingly demonstrate that activity at the GABA receptor is required for the pediculicidal activity of thymol. As the evidence so far suggests that there is no chemical compound to which insects cannot become resistant, a strategy for confirming GABAR involvement might be to select for thymol resistant strains of lice in the laboratory. DNA sequences from wild-type lice, mutant lice and *Drosophila* could then be compared in order to identify if the mutant genes encoded GABA receptor subunits.

#### ***Lethal effects of monoterpenoids on lice: the next stages of experimental development***

Although neuronal targets like the GABAR have been identified as causing the rapid symptoms of insecticide poisoning or 'knock-down,' it has been suggested that insects can recover from certain types of neuronal paralysis [94], and a lack of correlation between knock-down and mortality has been demonstrated in some cases [91]. It seems that mortality and disturbances in motor neuron activity are not as tightly linked as many insecticidal studies assume.

Interestingly, recent evidence suggests that insecticide action at neuronal receptors that control neurosecretion may be more important in determining death [61] than at those receptors affecting co-ordination and locomotion; the former is also considered irreversible. In one study, desert locusts from which the diuretic hormone producing *corpora cardiaca* had been removed, failed to undergo pyrethroid-induced water-loss, unlike the intact animals [97]. In this thesis, some monoterpenoids, especially cineole, induced a profound shrivelling effect in lice, however, these lice did not cease movement throughout the course of the experiment, leading them to be classified as alive. Although a paralysing effect on lice is beneficial, as writhing lice can be felt by the patient, the paralysis must be irreversible. Interestingly, a recent study identified anti-RDL staining in the *corpora cardiaca*, suggesting the subunit might function in the control of neurosecretion [221]; however, further proof would be required for the involvement of the GABAergic effect of monoterpenoids in water-loss, especially as antibody staining to RDL<sub>ac</sub> is fairly widespread in insect nervous systems.

This thesis has provided the first evidence of monoterpenoid and eugenol interaction at a specific, isolated, insect neuronal receptor target site: the insect GABA receptor. This may have implications not only with respect to monoterpenoid toxicity, but also the wide variety of other ecological effects they elicit in insects. Equally importantly, it provides evidence that combinations of monoterpenoids, such as those found in essential oils, may prove to be more effective at eradicating infestations of pediculosis capitis than a single entity applied alone; future formulations may not only include distinct ovicidal and pediculicidal actives, but also perhaps those with effects on different physiological systems, with the aim of maximising toxicity and minimising treatment failure and resistance.



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