# UNIVERSITY OF SOUTHAMPTON

## FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

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**The role of contact chemoreception in the egg-laying behaviour of the desert locust, *Schistocerca gregaria***


By

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# Abstract

Chemoreception plays an important role in the egg-laying behaviour of locusts. Chemicals that represented the basic taste classes were added to sandy substrates at various concentrations and their effect on egg-laying analysed. Concentration dependent reductions in egg-pod number were observed for all chemicals, with the highest concentrations of all chemicals preventing egg-laying. Similarly, locusts spent less time egg-laying in substrates containing higher chemical concentrations, compared to low chemical concentrations. The concentration at which chemicals acted aversively depended on the type of chemical.

Isolation of the locust abdomen from the thorax produces fictive digging movements of the ovipositor valves that are required for successful egg-laying. Chemical stimulation of the ovipositor valves of rhythmically active preparations resulted in a cessation of the digging rhythm. High chemical concentrations stopped the digging rhythm for significantly longer durations than low chemical concentrations and distilled water controls.

The neuromodulator, nitric oxide (NO) was found to modulate the oviposition digging rhythm. Increasing and decreasing NO and its known molecular targets in the part of the nervous system that produces the digging movements of the valves, the terminal abdominal ganglion, significantly increased and decreased the frequency of the digging rhythm respectively. Moreover, NO was also found to significantly alter the effects of chemical stimulation of the valves. Increasing NO levels in the terminal abdominal ganglion resulted in a significant decrease in the duration of cessation of the digging rhythm when the valves were chemically stimulated, whereas decreasing NO levels resulted in a significant increase in the cessation of the digging rhythm.

This study shows that the chemical composition of the substrate is a determinant of choice of oviposition sites and egg-laying success, and that NO can regulate the effects of chemosensory input on behaviour.

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# Chapter 1 – General Introduction

## Chapter 1

# General Introduction

The sense of taste, contact chemoreception or gustation as it is also commonly known, has a central role in the life of all animals. Together with olfaction (the sense of smell - the detection of airborne molecules) both senses function to assess food quality, to regulate its ingestion (Montmayeur an d Matsunami, 2002; Chapman, 2003) and to prepare for digestion (Spector, 2000). In terrestrial insects, contact chemoreception underlies a diverse range of other behaviours including mate location and selection (Ruther *et al.* 2000; Masante- Roca *et al.* 2002), host plant location and selection (Jallow *et al.* 1999; Degen and Stadler, 1996; Degen and Stadler, 1997), avoidance behaviour (Rogers and Newland, 2000; Newland, 1998) and the selection of suitable egg-laying sites (Woodrow, 1965; Uvarov, 1977; Popov, 1980), all of which would be impossible without a well developed chemosensory sense.

Egg-laying is one of many complex tasks an insect must accomplish, requiring the integration of many distinct external and internal sources of sensory information and the appropriate coordination of several types of motor response (Facciponte and Lange, 1996). The rules governing the discrimination between suitable and unsuitable egg-laying sites have been investigated extensively at the behavioural level in many species of insect (Degen and Städler, 1996; Jallow *et al.* 1999; Woodrow, 1965). The integration of chemosensory information within both the peripheral and central nervous system is thought to make an important contribution to the neuronal processes underlying this behaviour (Baur *et al.* 1998).

Anatomical and physiological analyses of the peripheral chemosensory systems have been performed on several species of Orthopterans and many other insect species

(Baur *et al.* 1998; Urvor *et al.* 1981; White and Chapman, 1990), and we now know much about how chemicals are detected and where chemosensory neurones project in the central nervous system (Newland *et al.* 2000; Tousson and Hustert, 2000). By contrast, very little is known of the central processing of contact chemosensory information, despite having a more immediate role in the selection of egg-laying sites.

## 1.1 Chemosensory sensilla, their locations and their organisation

Insects detect chemicals by means of small, peg-like, cuticular chemosensitive sensilla that are scattered in varying densities over the surface of most insects (Chapman, 1982). These sensilla, termed basiconic sensilla, are uniporous and range from 10 - 50μm in length (Blaney, 1981). In contrast to olfactory sensilla, basiconic sensilla are innervated by between 2 and 10 chemosensory neurons and one mechanosensory neuron, along with supporting cells, located beneath the cuticle at the base of a sensillum. The sensory dendrites of the chemosensory neurons project from the cell bodies into the fluid filled lumen of the sensillum (Dethier, 1976). The number of basiconic sensilla varies both within and between species and can range from tens to thousands over the entire surface of the body (Chapman, 1982). This variation often reflects the dietary breadth of an insect. For example, polyphagous insects (insects with a highly variable diet) have more sensilla than oligophagous insects (insects that feed on a low number of plant species), that in turn have more than monophagous insects (insects that feed on one species of plant only) (Greenwood and Chapman, 1984). Sensilla number also varies with the size, age and sex of an insect (Rogers and Simpson, 1997). The numbers and densities of basiconic sensilla are highest on areas that most frequently come into contact with chemicals, including the antennae, mouthparts, hind legs and the ventral surface and tips of the ovipositor valves. Basiconic sensilla attach to the cuticle by a flexible socket and are bi-modal; responding to tactile and chemical stimulation (Newland and Burrows, 1997). The

antennae of insects are predominantly populated by olfactory multiporous sensilla whose overall structure and shape can vary enormously. Most take the form of outwardly protruding hairs or pegs, the entire surface of which can be porous. Some contact chemosensory sensilla can be present on the antennae in the form of indented pits (Greenwood and Chapman, 1984).

In the olfactory system, sensory neurones that respond to odour stimulation project to anatomically distinct parts of the antennal lobe neuropil, termed glomeruli (Ignell *et al.* 2001). Different odours can excite different combinations of glomeruli, the activity of which overlap, producing spatial codes of both individual and blends of odours (Menzel *et al.* 2005). This has led to the use of the term odotopic organization for the way in which olfactory afferents project centrally. By contrast, there seems to be no anatomically distinct centre for gustatory processing (Newland *et al.* 2000; Tousson and Hustert, 2000).

Contact chemosensory afferents are organised somatotopically within the central nervous system in such a way that the location of a basiconic sensillum in the periphery is reflected by the projection of its chemosensory neurones in the central nervous system (Newland *et al.* 2000). The locust is therefore provided with information about the precise location of a chemical stimulus on its body.

## 1.2 Egg-laying behaviour

Insects select egg-laying sites principally from sensory information from its peripheral chemosensory system. This information is then integrated within the central nervous system and an appropriate motor, and therefore behavioural output produced. On encountering a potential egg-laying site, Orthopteran insects will often sample the substrate with their mouthparts and antennae (Uvarov, 1977). A period of tarsal contact is also observed before the locust either moves away from the substrate, or proceeds to rhythmically sample the substrate with the tips of its ovipositor valves

(Popov, 1958a). Should the substrate prove to be unsuitable for egg-laying, Orthopterans will stop sampling and sample alternative substrates. If the substrate is not rejected, rhythmic sampling movements become rhythmic digging movements (Uvarov, 1977). In locusts, as in most insects, this behavioural chain is not fixed. A potential egg-laying substrate can be rejected at any stage, even after digging of the substrate has commenced. Individuals may also dig potential egg-laying substrates without any of the prior sampling behaviours (Norris, 1968).

## 1.3 Transduction of contact chemosensory stimuli

When the ovipositor valves contact a potential egg-laying substrate, chemical stimuli diffuse across the fluid filled lumen of the basiconic sensillum, where it is detected by receptors within the membranes of the dendrites (Hodgson *et al.* 1955). Although it is not the focus of this thesis, an understanding of taste transduction provides a basis for understanding the effects of neuromodulators on chemosensory processing and the generation of rhythmic movements.

The subject of taste transduction is complex (for recent reviews see Lindemann, 2001; Margolskee, 2002) and studies have largely focused on isolated taste receptor cells (TRC's) of rodents. This is mainly because of the rapid turnover of TRC's (making them readily available for study), the average lifespan being approximately 10 days and their large size (30-40μm) (Bigiani *et al.* 1997) compared to TRC's in other model organisms. Few studies have been performed on transduction in locusts, and what has been done has focused purely on the transduction pathways of bitter tasting stimuli (Glendinning and Hills, 1997; Glendinning *et al.* 1999, 2002). For this reason, most of this section will refer to research focusing on vertebrates.

The first encounter an insect makes with molecules is at the level of the contact chemosensory receptors, which provide the specificity of the chemosensory response.

In *Drosophila*, however, odorant binding proteins (OBP's) have been identified in taste tissue that could provide the initial binding sites for chemical stimuli (Koganezawa and Shimada, 2002). This suggests that in *Drosophila*, some chemosensory sensilla may have a dual function, mediating both olfaction and contact chemoreception. This also provides the possibility that binding proteins may be present in the fluid filled lumen of Orthopteran taste receptors that bind to molecules prior to their transduction. Recent evidence has shown that a number of binding proteins are present in the fluid filled lumen of blowfly (*Phormia regina*) basiconic sensilla, although calmodulin is the most abundant (Seno *et al.* 2005). When basiconic sensilla are stimulated with sucrose and a calmodulin inhibitor, W-7, the impulse frequency from sucrose responsive cells decreases with increasing concentrations of W-7. These results suggest that calmodulin acts as a binding protein for sucrose molecules in the initial stages of sucrose taste transduction.

In insects, contact chemosensory receptor proteins are located on the dendrites that project from the sensory neurons into the shaft of the sensillum (Glendinning *et al.* 2001). The diversity of contact chemosensory receptor proteins is immense, including ion channels, enzymes and G-protein coupled receptors (GPCR's) (Roper, 1983; Avenet and Lindemann, 1987).

**Salt**: Salt taste guides the incorporation of sodium chloride and other required minerals into the body and serves the function of ion and water homeostasis (Lindemann, 2001). Salt taste transduction involves both sodium-specific, and non-specific, mechanisms and was discovered by blocking a highly selective sodium channel, epithelial sodium channel (ENaC), with the sodium channel blocker amiloride (Canessa *et al.* 1994). ENaC is a hetero-oligomeric complex (comprising three homologous sub-units) and acts as a salt taste receptor by providing a specific pathway

for the passage of a sodium current into taste cells (Garty and Palmer, 1997), that triggers action potentials (via the receptor potential).

**Sour**: In many species, the detection of sour taste serves to detect spoiled, acid damaged food (Lin *et al.* 2003). The transduction of sour taste primarily involves the detection of extracellular $H^+$ ions (Lyall *et al.* 2001). A variety of ion channels are involved in sour taste transduction, that also includes the ENaC family of channels, which can mediate inward proton currents and $H^+$-gated potassium channels, specifically hyperpolarization-activated, cyclic nucleotide-gated cation channels (Desimone *et al.* 1995). These are simply two examples of a variety of mechanisms found for sour taste that highlight the complexity of taste transduction. Taste transduction for both of these simple ions involves the altered permeation of the receptor cell membrane and resulting receptor currents in TRC's stimulate neurotransmitter release to excite sensory afferents (Chaudhari *et al.* 2000).

**Bitter**: Bitter taste can, but need not, be an indicator of toxicity and in vertebrates is detected by a taste specific G-protein called gustducin (Caicedo *et al.* 2003). Knockout mice lacking α-gustducin have been shown to have a decreased sensitivity to bitter agents such as quinine, and interestingly for sweet tasting compounds such as sucrose (Lindemann, 2001). Another transduction cascade involves a GPCR mediated bitter signal triggering a decrease of intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) which is accompanied by increases in inositol-1,4,5, triphosphate ($IP_3$). This results in the activation of $IP_3$ - sensitive intracellular calcium stores and concomitantly, calcium ions are released into the cytosol (Akabas *et al.* 1988; Bernhardt *et al.* 1996). The subsequent effect on channels responsible for alterations in membrane potential however, remains to be identified (Lindemann, 2001).

What is not known is whether both types of transduction cascade are required for bitter taste transduction, or whether they are required for bitter taste discrimination i.e. the discrimination between toxic and non-toxic bitter tasting compounds (Glendinning *et al.* 2002).

**Sweet**: Studies in insects have shown that sweet taste transduction is also mediated by GPCR's (Chyb *et al.* 2003). An identified gene, *Gr5a*, was identified and cloned in *Drosophila*, and found to encode a trehalose receptor and its presence essential for the sensory responses to sugar (Chyb *et al.* 2003). In vertebrates, sweet responsive cells have been shown to use both cyclic nucleotides and $IP_3$ as second messengers during the transduction process. Calcium imaging experiments with isolated vallate taste buds of rats showed that stimulation of the taste cells with sucrose caused calcium uptake from the extracellular space (Bernhardt *et al.* 1996). Conversely, non-sugar sweeteners cause calcium release from intracellular stores. This shows an unusual parallel with bitter taste transduction in that two pathways seem to be involved in detecting sweet tastes.

**Amino acids**: Analyses of the taste transduction pathways of amino acids have mainly focused on the sensation of glutamate. This is due to glutamate's ubiquity in proteinaceous human food including meat, fish, cheese and some brassicae plants. Glutamate is also found in various other forms such as the flavour enhancer, monosodium glutamate, and receptors for glutamate have been characterized and identified within the peripheral and central nervous system of all animals (Dingledine and Conn, 2000). The taste sensation of glutamate is qualitatively different to those of sweet, salty, sour and bitter taste sensations, and signals protein rich food (Ikeda, 1909) and has led to the use of the descriptive term 'umami' derived from the Japanese for delicious 'umai' in reference to glutamate sensation.

Taste receptors for glutamate may be related to a type of glutamate receptor well known from neuronal synapses (Chaudhari *et al.* 2000). This follows the discovery of a subset of taste cells that contained the truncated form of mGluR4, a metabotropic glutamate receptor abundant in the central nervous system (Corti *et al.* 2002). Transduction of glutamate is also complex. Studies have shown that an unspecified cation channel shows a sustained closure behaviour leading to the hyperpolarization of the taste cell membrane (Bigiani *et al.* 1997). A similar effect of glutamate on TRC's was found by Bigiani *et al.* (1997), although sustained hyperpolarizing responses were punctuated by transient depolarizations, and to add to the complication of taste transduction research, were found to be sodium dependent (Bigiani *et al.* 1997). This fact alone has led to conjecture over whether glutamate taste sensation is separate from that of sodium as postulated by Lindemann (2001).

Taste transduction is therefore a highly complex process involving the initial binding of taste molecules with membrane receptors and the triggering of downstream transduction events, such as the activation or inactivation of intracellular second messengers. This in turn alters the intracellular ionic environment of the cell sufficiently to affect the membrane potential of the taste receptor cell and evoke a depolarizing or hyperpolarizing response. The resulting influx and efflux of different ions (the inward or outward currents), produces action potentials or inhibition with transient episodes of depolarization. These action potentials are then propagated to, and integrated within, the central nervous system.

## 1.4 Chemosensory coding

Once transduced, information relating to chemical quality must be encoded in the nervous system. The specificity of chemoreceptors is of great contention and at both the peripheral and central level, chemoreceptors need not only respond to chemicals of behavioural relevance. Such is the lack of information on contact

chemosensory coding, most research to date has focused on the peripheral and central coding of individual chemicals that are of behavioural relevance.

Although it seems likely that insects can detect and recognise different compounds, natural behaviours often require the detection and recognition of many chemicals, at various concentrations and in a range of mixtures. This has led to two theories as to how chemical stimuli are encoded in the peripheral and central nervous system. One theory proposes that distinct neuron classes each code for a different taste quality, the so-called labelled line theory, and assumes that there are a finite number of chemicals to which an animals chemosensory system can respond (Pfaffmann, 1959). Alternatively, the second theory called across-fibre-patterning, proposes that individual chemosensory neurons each respond to a number of different chemicals and with the range of sensitivity of any individual neuron overlapping with that of several others (Pfaffmann, 1974).

Recordings from the taste receptors of Dipterans have shown that individual peripheral contact chemosensory neurons respond to distinct classes of chemical stimuli. In *Drosophila* it has been shown that there are four different contact chemosensory neurons. A sugar sensitive (S) neuron responds maximally to most sugars and some amino acids, an L1 and L2 neuron that respond to salts and anions respectively, and the 'W' cell that detects the presence of water (Dunipace *et al.* 2001; Pollack and Balakrishnan, 1997). In caterpillars an additional deterrent cell, or 'D' cell, responds to secondary plant compounds and is correlated with aversion responses (Schoonhoven and van Loon, 2002). It has since been suggested however, that because the chemicals encountered by *Drosophila* (in a contact chemosensory context) are often part of a mixture, across-fibre-pattern coding applies. Subsequently, it was discovered that L2 sensory neurons were also able to respond to bitter stimuli, further supporting the across-fibre-coding theory in the peripheral nervous system of insects

(Meunier *et al.* 2003). Similarly, in the diamondback moth, *Plutella xylostella,* one sensory neuron can respond to seven different glucosinolates, all of which are found (among others) in various concentrations in different species of Brassicae (van Loon *et al.* 2002). Chemicals normally encountered by insects would be in the form of blends, and so the combined activity of the many neurons would reflect both the behavioural relevance of the compound and a total qualitative assessment of behaviourally relevant chemicals that the blend contains (de Jong and Städler, 2002).

Peripheral coding of contact chemosensory stimuli in the locust, therefore appears to be based on an across-fibre-pattern and without distinct neuron classes that respond separately to salts, carbohydrates and amino acids. The concentration of a chemical stimulus is encoded by the frequency of action potentials (White and Chapman, 1990). Increasing concentrations of a given chemical stimulus evoke higher frequencies of action potential, and often the activity of more than one sensory neuron, than lower concentrations of a chemical stimulus.

Recordings from the peripheral nervous system of locusts have also shown that sensory neurons adapt rapidly to contact chemosensory stimuli (Maes and Harms, 1986). The number of action potentials evoked in a sensory neuron decreases as the duration of the recording is increased. The adaptation is not permanent and action potentials can be evoked after a period of recovery (White and Chapman, 1990). It is much less clear as to how contact chemosensory information is represented in the central nervous system. Recordings from individual spiking local interneurons in the metathoracic ganglion of the locust have shown a similar relationship between chemical concentration and numbers of action potentials evoked (Rogers and Newland, 2002). Increasing concentrations of sodium chloride (NaCl), sucrose, nicotine hydrogen tartrate (NHT) and lysine glutamate (LG), were shown to increase the numbers of action potentials evoked from spiking local interneurons, which in turn

were significantly correlated with neural and therefore behavioural output (Rogers and Newland, 2002).

During the egg-laying behaviour of locusts, the entire surface area of the ovipositor valves is in contact with the substrate. Assuming that the central projections of contact chemosensory afferents are somatotopic, sensilla across the whole surface of the ovipositor valves will be communicating the chemical composition of the substrate to different interneurons in different regions of the central nervous system. Presumably the pattern of activity across populations of interneurons within the central nervous system has a role in the decision making process that either accepts or rejects a potential food or egg-laying substrate.

Although Tousson (2001) has shown that different classes of interneuron can respond to chemosensory stimulation, including local interneurons (neurons that lack an axon projecting out of the terminal abdominal ganglion) and intersegmental interneurons (neurons with their cell body located in one ganglion, but with axons projecting to another ganglion via a connective), it is not known how or which of these interneurons contribute to the maintenance and output of the digging movements of the abdomen. Spiking local interneurons however, are responsible for the integration of many sensory inputs including mechanoreception and inputs from campaniform sensilla that monitor cuticular stress (Newland, 1999). The central processing of contact chemosensory stimuli is therefore not distinct from the processing of other sensory modalities such as the central processing of mechanosensory information.

## 1.5 Modulation of contact chemosensory responses

Our limited knowledge regarding the central processing of contact chemosensory information within local circuits has resulted in a lack of knowledge as to how contact chemosensory stimuli and resultant motor output are modulated. The behavioural output of a network is not rigidly defined and animals need to modify the

motor output of a given neural network depending both on the animals current physiological state and when responding to cues from the external environment. A certain degree of plasticity within the central nervous system of the animal is therefore required. Locusts for example can regulate their response to a given food type, depending upon its nutritional state (Behmer *et al.* 2001). Recordings from basiconic sensilla on the maxillary palps of locusts have shown that the peripheral nervous system of protein deficient locusts, responds more vigorously when presented with an amino acid stimulus than when protein replete. Likewise, depriving a locust of carbohydrate results in a vigorous response from chemosensory neurons when exposed to sucrose (Simpson and Raubenheimer, 1996).

Similarly, the choice of egg-laying sites requires the central nervous system to recognise suitable from unsuitable egg-laying substrates, but also to make the appropriate motor output to commence or terminate digging of the substrate. Egg-laying in insects is also subject to other confounding factors. For example, in butterflies the choice of egg-laying sites varies with the egg-load of the insect. Female butterflies are less discriminatory when their egg-load is particularly high, often accepting the first substrate (irrespective of its suitability) they encounter on which to lay their eggs (Hopkins and van Loon, 2001). Unpredictable environments can simply fail to offer any suitable egg-laying sites. The neural networks responsible for the selection of egg-laying sites in a female insect must also have the plasticity to distinguish between the most suitable, and unsuitable, egg-laying substrates in the absence of the ideal.

The neural networks controlling the movements of various appendages are often confined to a local ganglion or ganglia, requiring minimal processing within higher centres of the nervous system (Mitchell *et al.* 1999). The neural networks controlling the movements of the ovipositor valves in locusts, are also endogenously

rhythmic, producing their movements without external sensory input (Facciponte and Lange, 1996). This has led to the use of the term 'central pattern generator' (CPG) (Brown, 1911; Delcomyn, 1985). Together with the realization that CPG's are flexible, and are often loosely coupled to other neural networks, the question has been posed of how plasticity arises. Various neuromodulatory substances have been found to modify the output of a CPG by modifying the connections between certain neurons that are part of the neural network.

Biogenic amines, peptides and gases have all been found to modify the output of various types of CPG, as well as initiate and terminate motor patterns (Rast and Braunig, 2001; Rast, 2001), and produce switching from one CPG to another (Simmers and Bush, 1983). Many of these neuromodulatory substances are also involved in contact chemosensory responsiveness. For example, stimulating the lips of molluscs with sucrose has been shown to increase levels of NO around the buccal ganglion controlling feeding movements (Kobayashi *et al.* 2000), suggesting that NO may be involved in initiating rhythmic feeding patterns (Rast, 2001) and also involved in learning and memory processes, affecting future food choices (Katzoff, *et al.* 2002). Moreover, NO has a ubiquitous presence in the peripheral and central nervous system of both invertebrates and vertebrates, and is therefore thought to play a significant role in the modulation of various sensory inputs including contact chemoreception.

A major part of this thesis therefore attempts to elucidate the rules governing the behavioural responses and their modulation to chemosensory stimulation. This study initially focuses on the effects of chemicals, representing the basic taste qualities (carbohydrate, protein, salts and bitter tasting compounds), on the locust's choice of oviposition sites (see *Chapter 2*).

Locusts lay eggs by rhythmically digging appropriate substrates with appendages called ovipositor valves. A detailed study was carried out identifying the

structures and the muscle groups involved in producing the rhythmic abdominal movements. This then allowed an extracellular physiological analysis of the activity of these muscles (*Chapter 3*). The rhythmic abdominal digging movements can be reliably produced by isolating the abdomen from the rest of the locusts body (Thompson, 1986a). To determine if these ovipositor movements are influenced by chemical stimulation, chemicals were applied directly to the distal part of the isolated abdomen, and the effect on the rhythm analysed (*Chapter 4*).

Long term recordings of the opener muscles allowed an analysis of the effects of the neuromodulator NO, on the motor output of the oviposition rhythm (*Chapter 5*) to attempt to understand how the long term, dynamic modulation of the rhythmic behaviour can be made. Chapter 6 then focuses on the role of NO in modulating the behavioural responses of the oviposition rhythm to chemical stimulation to again identify possible modulatory mechanisms underlying chemoreception. The overall findings of this study are finally discussed (*Chapter 7*).

# Chapter 2 – The choice of egg-laying sites: the role of contact chemoreception

Chapter 2

# The choice of egg-laying sites: the role of contact chemoreception

## 2.1 Introduction

Contact chemoreception plays an important role in the location and selection of egg-laying (oviposition) sites by insects, including insect families that pose significant economic impact (Van Huis, 1995). These include Diptera (Degen and Städler, 1996; 1997; Barker, 1992; Levinson *et al.* 2003; Feder *et al.* 1997), Orthoptera (Woodrow, 1965; Uvarov, 1977; Popov, 1980; Hunter and Elder, 1999), Lepidoptera (Baur *et al.* 1998; Jallow *et al.* 1999; Hora and Roessingh, 1999) and Hymenoptera (Kuhlmann *et al.* 1998; Nurindah *et al.* 1999). It has also been shown that the choice of an egg-laying site often reflects a food choice. Female Lepidoptera, for example, select egg-laying sites (host plants) that both they and their larvae preferentially feed upon (Baur *et al.* 1998). Flies have also been observed to exhibit various feeding related sampling behaviours on encountering a potential egg-laying site (Degen and Städler, 1996). Drumming or scraping of the tarsi, palpating and biting movements of the mouthparts and probing movements of the antennae and egg-laying apparatus, the ovipositor, have all been described before the decision to accept or reject an egg-laying site is made (Baur *et al.* 1998).

Although it was realised by Zakharov (1927) that the suitability of an insect's egg-laying site depended on its chemical composition, Woodrow (1965) showed that chemicals representing anionic, ionic and bitter taste qualities influenced the choice of a locust's egg-laying site. More recently, Tousson and Hustert (2000) confirmed that the sensory neurons of basiconic sensilla, the contact chemoreceptors, located on

the locusts ovipositor, respond physiologically to a variety of chemical stimuli, including chemicals that represent the basic taste qualities (carbohydrates, salts, bitter and sour tastants, and proteins).

In an ecological context locusts have been shown to lay their eggs in moderately compact damp sand or soil (Popov, 1980), and sequences of different behaviours are observed before the decision to accept or reject an egg-laying site is made (Uvarov, 1977). On encountering a potentially suitable egg-laying site, female locusts are often observed to feed upon the substrate whilst simultaneously stroking the substrate with their antennae, and subsequently will then contact the substrate with tarsi of the fore-legs (Kennedy, 1949). Providing the egg-laying substrate has been deemed suitable, gravid females then raise their whole body to a stilted position (Clark, 1965) and arch their entire abdomen at an angle of approximately 90° to the rest of their body. The substrate is then gripped with the thoracic limbs while the hind legs are cocked in preparation for a defensive kick (Thompson, 1986a). A final sampling period is then observed that consists of probing, opening and closing movements of the ovipositor valves (Uvarov, 1977). These movements superficially sample the surface of the substrate and provide the locust with information regarding the chemical and physical properties of the substrate. Should the substrate be accepted at this stage, the cyclic opening and closing movements of the ovipositor valves are accompanied by protractive and retractive movements that serve to elongate the abdomen whilst simultaneously driving it into the substrate (Vincent and Wood, 1972). During this period, the repetitive protractive-retractive movements elongate the abdomen by up to 4 times its original length, from 2.5-3cm to 9-12cm (Vincent and Wood, 1972; Jorgensen and Rice, 1983; Rose *et al.* 2001). This behavioural strategy is unique to Orthopterans and serves to lay the locusts eggs at

depth, protecting them from desiccation, parasitization and predation (Jorgensen and Rice, 1983).

Once the hole has been excavated the valves of the ovipositor enter a prolonged gaping phase that subsequently activates rhythmic, myogenic contractions of the oviducts that serve to expel between 30-100 eggs into the hole (Kalogianni and Pflüger, 1992; Yi and Gillott, 2000). Following egg-laying the valves then close and the abdomen is slowly retracted back to its original length (Uvarov, 1977). This stage is accompanied by a secretion of foam by the accessory glands (Hagele *et al.* 2000) that serve to glue the newly laid eggs securely together in the form of a pod (Islam *et al.* 1994).

Various studies have questioned the importance of other potential factors that affect an animals behavioural response to chemicals. Taste related research has to date, shown that TRC's respond physiologically to the presence of a chemical via the activation of receptor proteins within the cell membrane that are able to detect that chemical (Bigiani *et al.* 1997). For example the sense of sour taste is linked to the detection of pH. As the pH of a tastant decreases, the degree of 'sourness' of that tastant increases (Lin *et al.* 2001). Ion channels in TRC's have recently been discovered that are pH sensitive and respond physiologically to the presence of extracellular $H^+$ ions (Bobkov and Kolesnikov, 1999). Similarly, the osmolarity of a solution such as a tastant, could potentially have an effect upon the TRC's with which the tastant comes into contact (Gilbertson, 2002). The osmolarity of a solution, when in contact with TRC's is known to affect the volume of the TRC's (Lyall *et al.* 1999). Studies on the effect of chemical stimuli on rat chorda tympani (CT) nerve responses have been shown that they are modulated by osmolarity (Lyall *et al.* 1999). The individual neurons of the taste receptors respond physiologically by depolarizing in the presence of NaCl. When the concentration of NaCl is kept

constant but the osmolarity of the solution manipulated, the degree to which the CT nerve depolarizes is affected. As the osmolarity of a solution is increased by the addition of a concentration of mannitol or cellobiose, the CT responses (the degree of depolarization) also increase. Changes in the osmolarity of a solution when in contact with cells of any type, affect that cells volume (Lyall *et al.* 1999). Consequently, this affects the pressure that is exerted on the cell membrane from the inside of the cell. In the case of neurons e.g. TRC's, mechanical stress upon the cell membrane potentially affects the way in which the TRC's are depolarized (Lyall *et al.* 1999).

This chapter describes an analysis of the role of contact chemosensory stimuli in a locust's choice of egg-laying sites. This is of importance because an understanding of how non-toxic chemicals potentially affect locust egg-laying behaviour, may lead to the discovery of non-toxic control measures of locust swarms. Moreover, understanding choice behaviours allows the further study of how these behavioural responses may be modulated. This study provided locusts with a choice between a chemically treated egg-laying substrate and an ideal control substrate. The osmolarity and pH of the above mentioned chemicals and their varying concentrations were also recorded.

## 2.2 Materials and Methods

### 2.2a Locusts

Female desert locusts, *Schistocerca gregaria* (Forskål) were taken from a colony maintained at the University of Southampton, reared under crowded conditions and fed daily on seedling wheat and oats. The colony was, on occasion, supplemented with female locusts obtained from Blades Biological Supplies (UK). Adult locusts were used in all experiments, aged from 9 to approximately 14 days post-moult. All adults were examined prior to use to ensure that the ovipositor valves and surrounding cuticle were intact and undamaged.

### 2.2b Behavioural choice experiments

Behavioural choice experiments were performed using 15 female locusts placed in an observation cage (39cm x 46cm x 38cm) (Fig. 2.1A). Locusts were provided with roosting sites (wire mesh) and food sources of seedling wheat and oats. Locusts were given a choice of 2 potential egg-laying substrates in the form of sand-filled pots (Fig. 2.1B).

Both contained dampened sand but with one of the pots treated with one of 7 test chemicals (see *test chemicals*). The ratio of distilled water and chemically treated distilled water to sand was 40 ml of solution to 130 ml of washed and oven dried sand. Both pots were located at the front of the cage for ease of viewing. Behaviour was recorded for approximately 15 hr overnight using a JVC TK-C136OB colour video camera and a JVC SRL910EK time-lapse video recorder. On termination of filming, locusts were removed and their wings clipped so as to avoid re-use in subsequent experiments and returned to the colony. The number of egg pods were then counted in both control and test pots. The results presented here are based on over 1500 hr of video recording. Measurements of the durations for which

individual locusts exhibited egg-laying behaviour were measured to the nearest second.

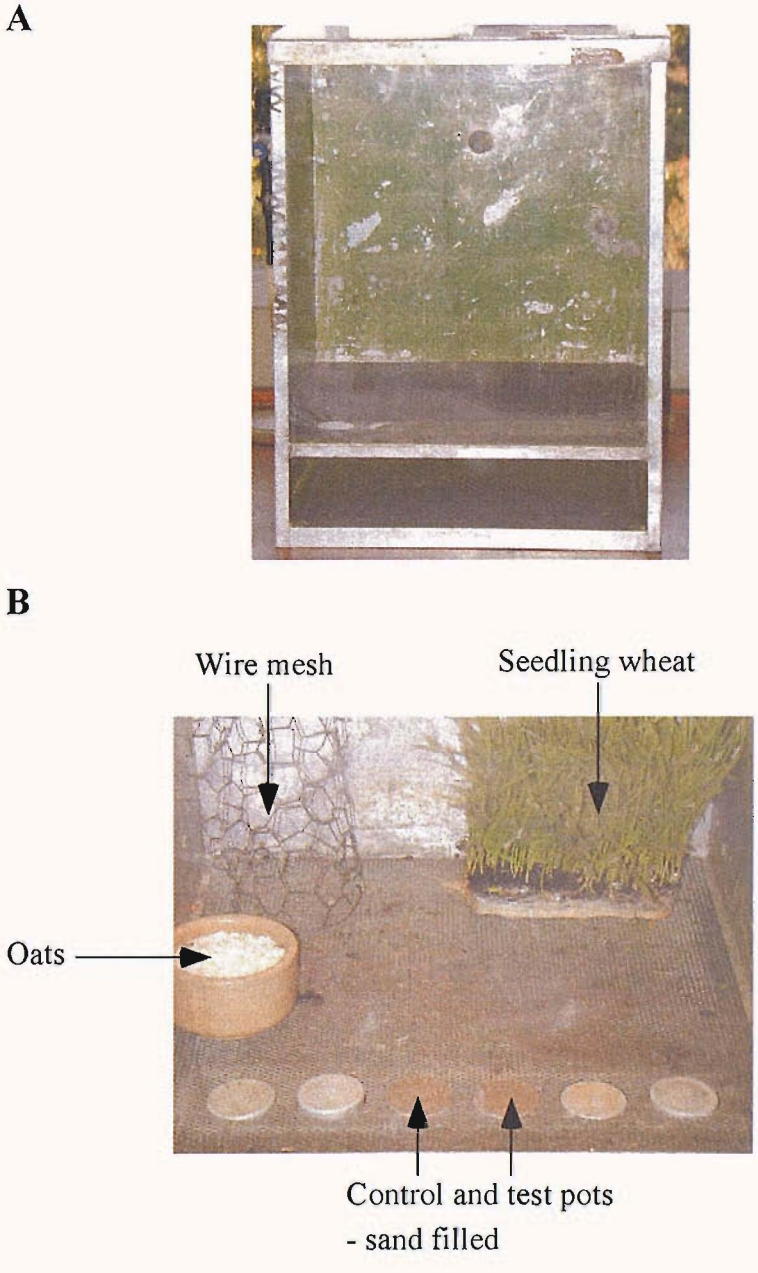



**Figure 2.1. The experimental set up for behavioural choice experiments. A.** An observation cage (dimensions = 39cm x 46cm x 38cm). **B.** During experiments female locusts were provided with food sources of seedling wheat and oats (to regulate dietary water intake) and wire mesh which acted as a roosting site. Locusts were provided with a choice of 2 egg-laying substrates; a control substrate of damp sand (sand mixed with distilled water) and a test substrate (of damp sand consisting of a concentration of 1 of 7 chemicals).

### 2.2c Test chemicals

The chemicals used in behavioural choice experiments were chosen based both on their relevance to locust behaviour and for their representation of basic taste qualities. NaCl and magnesium chloride (MgCl) both represent anionic, salt taste qualities. Sucrose and LG represent the macronutrients, carbohydrate and protein respectively and are components of the locusts' diet that are known to actively promote feeding (Simpson and Raubenheimer, 1993a). Hydroquinone (HQ) and NHT are bitter tasting, toxic anti-feedants (discouraging taste-related behaviour in insects) whereas tannic acid (TA) represents a sour tasting stimulus (increases $H^+$ ions).

The concentrations used varied for each chemical and the effective ranges were estimated by performing preliminary experiments. All chemicals were made up to their required concentration in distilled water. Test chemicals and their effective concentrations used in behavioural choice experiments were as follows: NaCl (Fisher Chemical Co); 75mM, 100mM, 250mM, 500mM and 1 M; MgCl (Sigma Aldrich Chemical Co): 25mM, 75mM and 250mM; Sucrose (Fisher Chemical Co): 250mM, 500mM, 1 M and 2 M; LG (Sigma Aldrich Chemical Co): 250mM, 500mM and 1M; HQ (Sigma Aldrich Chemical Co): 10mM, 25mM, 50mM, 100mM, 200mM and 500mM; NHT (Sigma Aldrich Chemical Co); 10mM, 25mM, 50mM, 100mM and 250mM and TA (Sigma Aldrich Chemical Co); 10mM 25mM and 75mM. The osmolarity and pH of all of the above solutions was determined using a CamLab Roebling Osmometer Automatik and Piccolo 2 ATC pH meter from HANNA instruments, respectively.

### 2.2d Statistical analysis

For all data obtained in the behavioural choice experiments, within chemical differences were analysed using one-way analysis of variance (ANOVA). Between

chemical differences were analysed for all parameters measured using analysis of covariance (ANCOVA) using $\log_e$ (chemical concentration) as the covariate. All statistical analysis was performed using SPSS v10.0 software for Windows.

## 2.3 Results

### 2.3a Effect of substrate composition on number of egg pods laid

When given a simple choice of a control substrate and one treated with chemicals, the number of egg pods laid in test pots decreased in a concentration dependent manner. The greater the chemical concentration, the lower the number of egg pods that were laid in the chemically treated substrates (Fig. 2.2). All chemicals used in the analysis stopped egg-laying at their respective higher concentrations. Chemicals representing those found in the locusts' diet tended to stop egg-laying at higher concentrations than anti-feedants. For example, locusts stopped laying eggs at a concentration of 2 M sucrose compared to a 75mM concentration of tannic acid.

Increasing concentrations of three out of the four chemicals representing a locust's dietary requirements resulted in significant decreases in the number of egg pods being laid in the test pots. For example, increasing LG from a concentration of 250mM to 1 M (Fig. 2.2B) resulted in a significant decrease in the number of egg pods laid in the test pot (ANOVA, $F_{3, 22} = 3.39$, $p < 0.05$) (Table 1). Similarly, increasing NaCl from a concentration of 75mM to 250mM (Fig. 2.2C) was sufficient to prevent egg-laying and resulted in a significant difference between the different concentrations (ANOVA, $F_{5, 34} = 5.86$, $p < 0.001$) (Table 1). Increasing the concentration of MgCl from 25mM to 250mM also significantly reduced the number of egg pods laid in test pots (ANOVA, $F_{3, 30} = 8.05$, $p < 0.0001$) (Fig. 2.2D). Although sucrose prevented egg-laying at its highest concentration (2 M), there was no significant, concentration dependent reduction in the number of egg pods laid in the test substrate (Fig. 2.2A). Increasing the concentration of sucrose from 250mM to a final concentration of 2000mM failed to produce a statistically significant

reduction in the number of egg pods laid (ANOVA, $F_{4, 23} = 2.12$, $p > 0.05$) (Table 1).

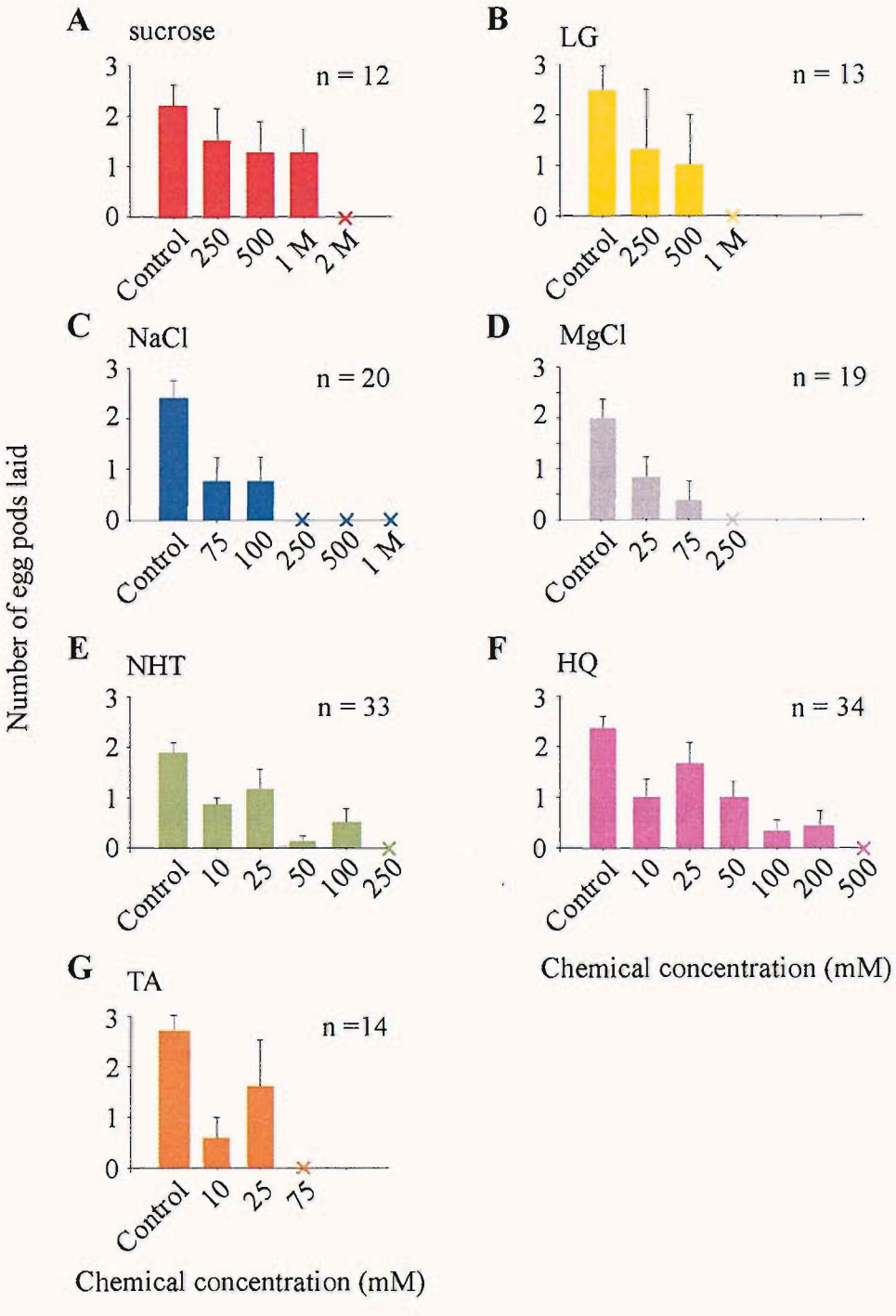



**Figure 2.2. The influence of chemical type and concentration on the number of egg pods laid.** Data are shown for **A.** sucrose, **B.** LG, **C.** NaCl, **D.** MgCl, **E.** NHT, **F.** HQ and **G.** TA. Values are means ± S.E.M. Data in each column are based on 4-6 experiments for each chemical test. Groups of 15 locusts were used for each experiment. The highest concentration of all chemicals tested prevented egg-laying in treated sand (marked with an 'x'). The reduction in the number of egg pods laid was significant ($p < 0.05$) for concentrations of LG, NaCl, MgCl, NHT, HQ and TA, but not for sucrose.

Increasing the concentration of anti-feedants also resulted in a concentration-dependent reduction in the number of egg pods laid in the test substrate. The anti-feedant NHT, in the concentration range of 10mM to 250mM (Fig. 2.2E), resulted in a highly significant reduction in the number of egg pods laid (ANOVA, $F_{5, 60} = 7.85$, $p < 0.0001$) (Table 1).

HQ acted in a similar manner (Fig. 2.2F), although over a slightly larger range of concentrations (10mM to 500mM), and produced a highly significant reduction in the number of egg pods laid in test pots (ANOVA, $F_{6, 61} = 6.18$, $p < 0.0001$) (Table 1). TA was highly effective in preventing egg-laying, stopping egg-laying at a concentration of as little as 75mM. Increasing the concentration of TA from 10mM to 75mM produced a statistically significant reduction in the number of egg pods laid in the test substrate (Fig. 2.2G) (ANOVA, $F_{3, 24} = 6.87$, $p < 0.01$) (see Table 1).

**Table 1: Analysis of variance of the effect of chemical type and concentration on the number of egg pods laid**. For 6 out of the 7 chemicals used, increasing the concentration of the chemical resulted in a statistically significant reduction in the number of egg pods laid ($p < 0.05$).

| Chemical | Sum of squares | d.f. | Mean squares | F | $p$ |
|---|---|---|---|---|---|
| sucrose | 14.833 | 4 | 3.708 | 2.123 | > 0.05 |
| LG | 22.173 | 3 | 7.391 | 3.389 | < 0.05 |
| NaCl | 46.8 | 5 | 9.36 | 5.861 | < 0.001 |
| MgCl | 28.431 | 3 | 9.477 | 8.047 | < 0.0001 |
| NHT | 37.341 | 5 | 7.468 | 7.848 | < 0.0001 |
| HQ | 47.987 | 6 | 7.998 | 6.176 | < 0.0001 |
| TA | 31.993 | 3 | 10.664 | 6.870 | < 0.002 |

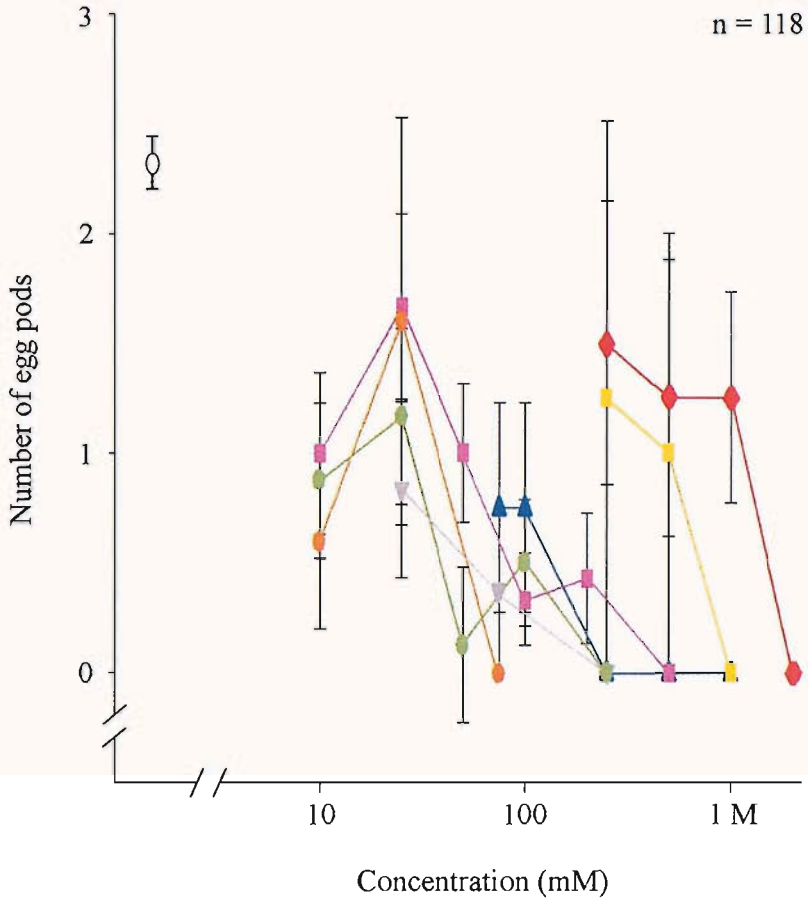



**Figure 2.3. Summary showing the effect of chemical type and concentration on the number of egg pods laid.** Data is shown for sucrose (red diamonds), LG (yellow squares), NaCl (blue triangles), MgCl (grey triangles), HQ (purple squares), NHT (green circles), and TA (brown circles). For each chemical the greater the concentration, the fewer the egg pods laid. Individual data points show the means and S.E.M. and are based on a total of 118 experiments with 15 locusts per experiment.

The results show that female locusts are able to make choices between suitable and unsuitable egg-laying substrates based on the chemical composition of the substrate. All locusts stopped laying eggs at the highest concentration of a particular chemical and the concentration at which a chemical acted aversively i.e. prevented egg-laying, depended on the identity of the chemical. For example,

sucrose prevented female locusts from laying eggs at a concentration of 2 M, compared to a concentration of 75mM for TA (compare Fig. 2.2A with 2.2G).

To test for statistically significant decreases in the numbers of egg pods laid, between all chemicals used, a statistical (ANCOVA) analysis was performed. (ANCOVA, $F_{10, 292} = 14.33$, $p < 0.0001$). This shows that all chemicals prevented egg-laying at their highest concentrations, and that statistically significant differences were observed between all 7 chemicals. Anti-feedants (e.g. TA and NHT) prevented egg-laying at lower concentrations compared to chemicals that are normally part of a locusts diet (LG and sucrose) (Fig. 2.3).

### 2.3b The effect of substrate composition on the duration of egg-laying

The effect of chemical type and concentration on the duration spent egg-laying was also analysed. All chemicals tested ultimately resulted in a concentration dependent reduction in the amount of time locusts exhibited egg-laying behaviour (with the abdomen submerged within a substrate). All chemicals that represented dietary requirements caused a statistically significant reduction in the duration that locusts spent egg-laying (ANOVA; sucrose, $F_{4, 70} = 2.81$, $p < 0.05$; LG, $F_{3, 67} = 3.82$, $p < 0.05$; NaCl, $F_{5, 251} = 58.37$, $p < 0.0001$ and MgCl, $F_{3, 44} = 3.99$, $p < 0.05$) (Fig. 2.4A-D) (Table 2).

All three anti-feedants used in the analysis (NHT, HQ and TA) also resulted in a statistically significant reduction in the duration of egg-laying (ANOVA; NHT, $F_{5, 218} = 19.27$, $p < 0.0001$, HQ, $F_{6, 315} = 22.09$, $p < 0.0001$, TA, $F_{3, 44} = 6.42$, $p < 0.0001$) (Fig. 2.4E-G) (Table 2).

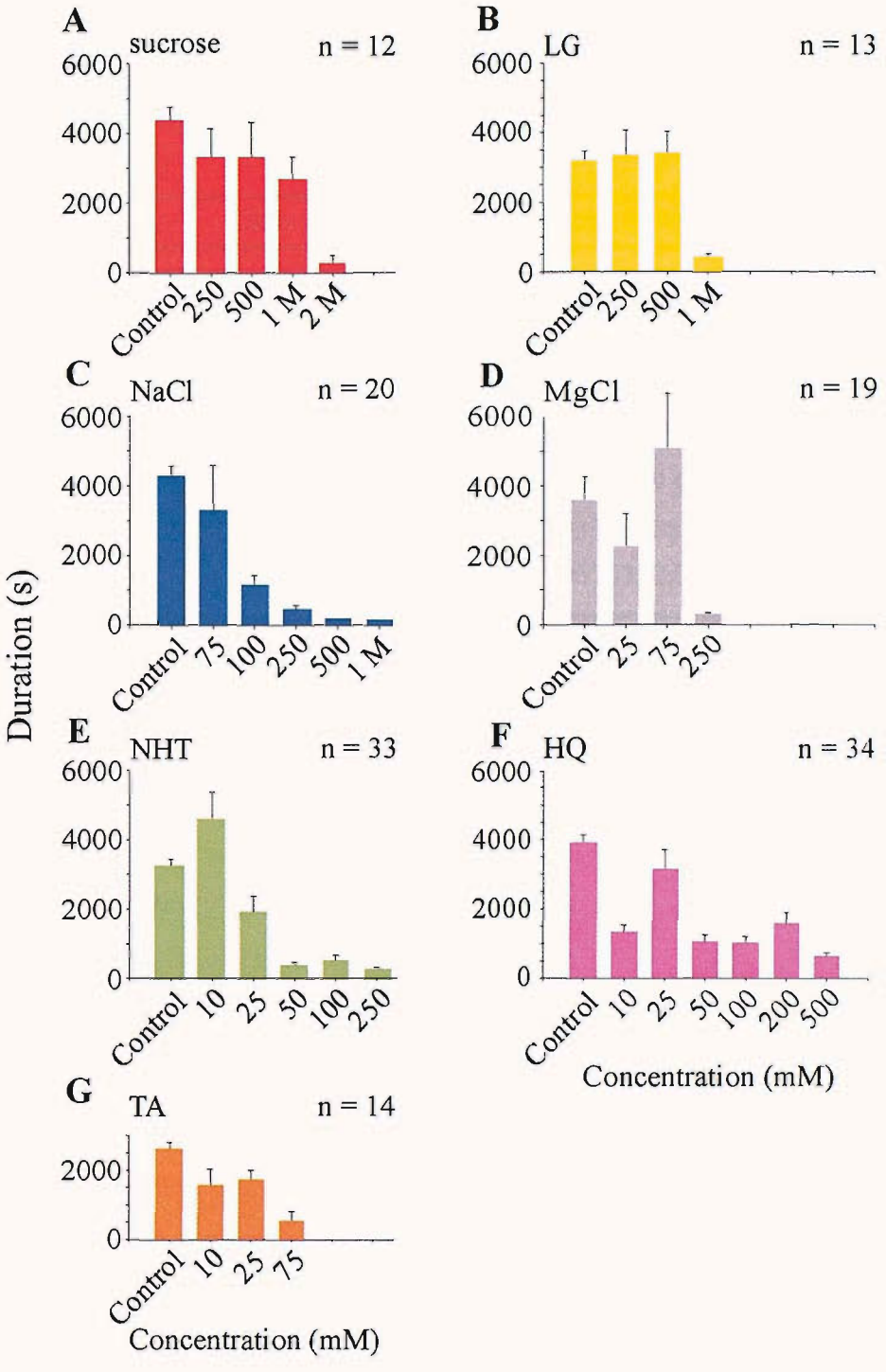



**Figure 2.4. The effect of chemical type and concentration on the duration of egg-laying.** Data is shown for **A.** sucrose, **B.** LG, **C.** NaCl, **D.** MgCl, **E.** NHT, **F.** HQ and **G.** TA. Values are mean ± S.E.M. Data in each column are based on 4-6 experiments for each chemical. 15 locusts were used for each experiment. All chemicals ultimately decreased the duration spent egg-laying in a concentration dependent manner ($p < 0.05$).

**Table 2: Analysis of variance of the effect of chemical type and concentration on the duration female locusts exhibited egg-laying behaviour.** Increasing the concentration of all chemicals resulted in a statistically significant reduction in the duration spent egg-laying ($p < 0.05$).

| Chemical | Sum of squares | d.f. | Mean squares | F | $p$ |
|---|---|---|---|---|---|
| sucrose | 79756804 | 4 | 19939200.908 | 2.806 | < 0.05 |
| LG | 49158856 | 3 | 16386285.381 | 3.823 | < 0.05 |
| NaCl | 895000000 | 5 | 179042157.67 | 58.372 | < 0.0001 |
| MgCl | 107000000 | 3 | 35641835.067 | 3.990 | < 0.05 |
| NHT | 403000000 | 5 | 80525709.684 | 19.272 | < 0.0001 |
| HQ | 620000000 | 6 | 103359088.29 | 22.09 | < 0.0001 |
| TA | 59011056 | 3 | 19670352.057 | 6.419 | < 0.0001 |

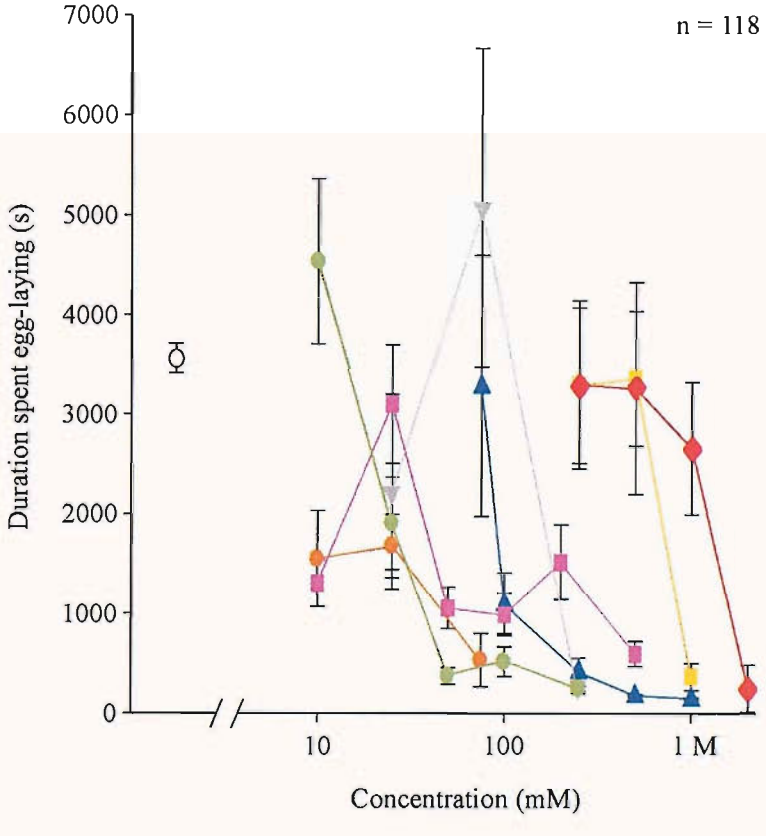



**Figure 2.5. Summary of the effect of chemical type and concentration on the duration of egg-laying.** Data is shown for sucrose (red diamonds), LG (yellow squares), NaCl (blue triangles), MgCl (grey triangles), NHT (green circles) and TA (brown circles). Distilled water control experiments are represented by the uncoloured circle. For each chemical, the greater the concentration the shorter the duration spent egg-laying. Individual points show means ± S.E.M. The data presented are based on a total of 118 experiments, each using 15 locusts.

In order to test for statistically significant differences in the duration locusts spent egg-laying between all chemicals, an ANCOVA analysis was performed. This produced a statistically significant difference between all chemicals used in the analysis (ANCOVA, $F_{10, 1171} = 37.08$, $p < 0.0001$) (Fig. 2.5), suggesting that the concentrations at which different chemicals act aversively depends on chemical type. Anti-feedant chemicals used in the analysis were again effective at lower concentrations compared to chemicals found in the normal diet.

### 2.3c The effect of substrate composition on the number of egg-laying attempts

The effect of chemical type and concentration on the number of egg-laying attempts was also analysed (Fig. 2.6). An egg-laying attempt was defined as any period of time that a female locust spends digging the substrate with its ovipositor valves. The concentration dependent effects observed with the number of egg pods laid and duration spent egg-laying, were not observed when analysing the effect of chemical concentration on the number of egg-laying attempts. Only 2 out of the 7 chemicals tested resulted in statistically significant differences between the different concentrations of a given chemical. Both the dietary requirement NaCl (Fig. 2.6C) and the anti-feedant NHT produced (Fig. 2.6E) statistically significant differences (ANOVA; NaCl, $F_{5, 34} = 5.14$, $p < 0.001$; NHT, $F_{5, 60} = 3.22$, $p < 0.05$) (Table 3) between chemical concentrations, but only NaCl showed a statistically significant, concentration dependent increase in the number of egg-laying attempts. All other chemicals tested in the analysis had no statistically significant effect on the number of egg-laying attempts (ANOVA; sucrose, $F_{4, 23} = 2.28$, $p > 0.05$; LG, $F_{3, 22} = 0.69$, $p > 0.05$; MgCl, $F_{3, 34} = 1.64$, $p > 0.05$; HQ, $F_{6, 61} = 1.13$, $p > 0.05$ and TA, $F_{3, 24} = 0.37$, $p > 0.05$) (Fig. 2.6A, B, D, F and H) (Table 3).

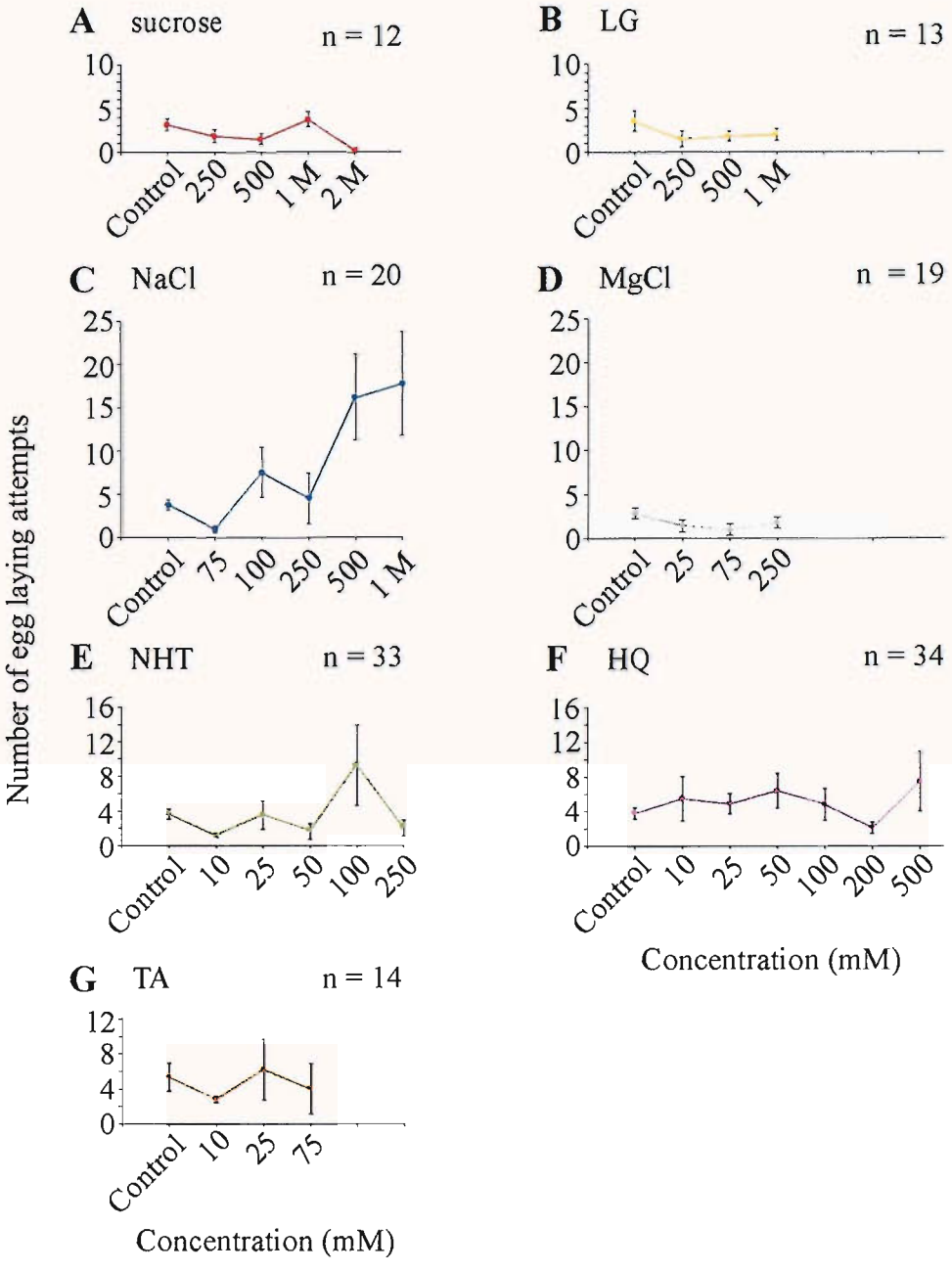



**Figure 2.6. The effect of chemical type and concentration on the number of egg-laying attempts.** Data is shown for **A.** sucrose, **B.** LG, **C.** NaCl, **D.** MgCl, **E.** NHT, **F.** HQ and **G.** TA. Individual points show means ± S.E.M. Increasing the concentration of NaCl resulted in a statistically significant increase in the number of egg-laying attempts ($p < 0.05$). Increasing the concentration of sucrose, LG, MgCl, NHT, HQ and TA did not have any significant effect ($p > 0.05$) on the number of egg-laying attempts.

**Table 3: Analysis of variance for the effect of chemical type and concentration on the number of oviposition attempts.** Increasing the concentration of 5 out of the 7 chemicals had no significant effect on the number of egg-laying attempts. Although increasing the concentration of NHT showed a statistically significant difference, there was not a concentration dependent increase. Increasing the concentration of NaCl resulted in a statistically significant increase in the number of egg-laying attempts.

| Chemical | Sum of squares | d.f. | Mean of squares | $F$ | $p$ |
|---|---|---|---|---|---|
| sucrose | 36.048 | 4 | 9.012 | 2.28 | > 0.05 |
| LG | 20.854 | 3 | 6.951 | 0.686 | > 0.05 |
| NaCl | 879.825 | 5 | 175.965 | 5.14 | < 0.001 |
| MgCl | 21.803 | 3 | 7.268 | 1.635 | > 0.05 |
| NHT | 216.712 | 5 | 43.342 | 3.223 | < 0.05 |
| HQ | 116.953 | 6 | 19.492 | 1.126 | > 0.05 |
| TA | 36.614 | 3 | 12.205 | 0.365 | > 0.05 |

### 2.3d Effects of substrate composition on the percentage of unsuccessful egg-laying attempts

The effect of chemical concentration on the percentage of egg-laying attempts that were unsuccessful was also analysed. For all chemicals tested the number of egg pods laid divided by the number of egg-laying attempts provided a measure of the percentage of egg-laying success. All chemicals tested in the analysis showed concentration dependent effects, with the percentage of unsuccessful attempts at egg-laying being greater the higher the concentration (Fig. 2.7). All seven chemicals showed statistically significant differences between their respective chemical concentrations (Table 4). All four dietary requirements showed statistically significant increases in the percentage of unsuccessful egg-laying attempts with increasing chemical concentration (ANOVA; sucrose, $F_{4, 23} = 4.24$, $p < 0.01$; LG, F

$_{3, 22} = 9.28$, $p < 0.0001$; NaCl, $F_{5, 34} = 8.81$, $p < 0.0001$; MgCl, $F_{3, 34} = 4.77$, $p < 0.01$)

(Fig. 2.7A-D).

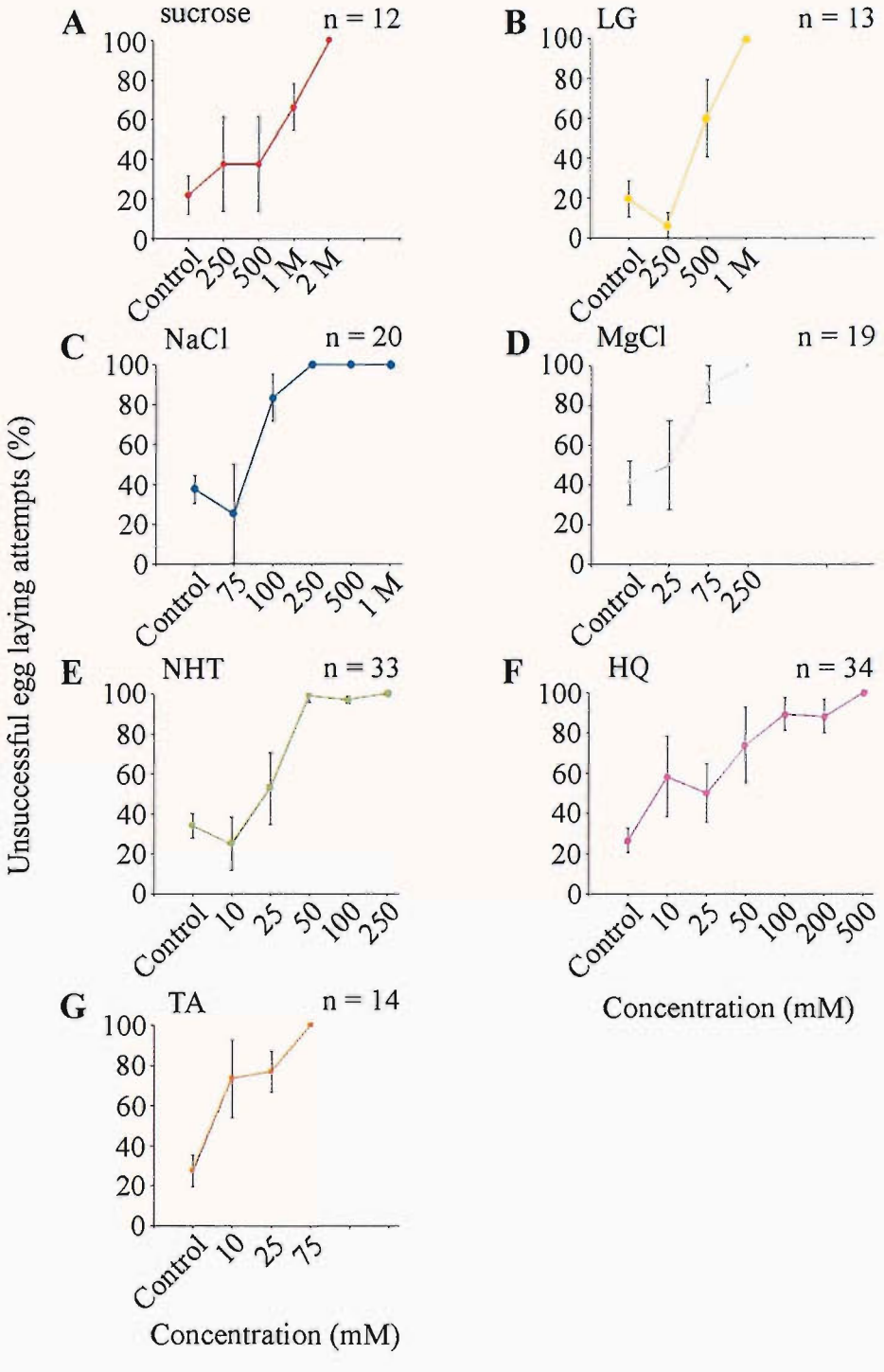



**Figure 2.7. The effect of chemical type and concentration on the percentage of unsuccessful egg-laying attempts.** Data is shown for **A.** sucrose, **B.** LG, **C.** NaCl, **D.** MgCl, **E.** NHT, **F.** HQ and **G.** TA. Values are mean ± S.E.M. Data in each column are based on 4-6 experiments. 15 locusts were used for each experiment. Statistically significant differences ($p < 0.05$) were found between all individual chemicals and their concentrations.

Increasing the concentration of all of the anti-feedants used in the analysis resulted in large increases in the percentage of unsuccessful egg-laying attempts. All three anti-feedants showed statistically significant increases in the percentage of unsuccessful egg-laying attempts between their highest and lowest chemical concentrations. Increasing the concentration of NHT, HQ and TA all resulted in statistically significant increases in the number of unsuccessful attempts at egg-laying (ANOVA; NHT, $F_{5, 60} = 11.34$, $p < 0.0001$ and HQ, $F_{6, 61} = 7.38$, $p < 0.0001$, TA, $F_{3, 24} = 8.61$, $p < 0.0001$) (Fig. 2.7E, F). (see Table 4).

**Table 4: Analysis of variance for the effect of chemical type and concentration on the percentage of unsuccessful egg-laying attempts.** Increasing the concentration of all 7 chemicals resulted in a statistically significant increase in the percentage of unsuccessful egg-laying attempts ($p < 0.05$).

| Chemical | Sum of squares | d.f. | Mean of squares | $F$ | $p$ |
|---|---|---|---|---|---|
| sucrose | 20375.447 | 4 | 5093.862 | 4.24 | < 0.01 |
| LG | 26318.056 | 3 | 8772.685 | 9.278 | < 0.0001 |
| NaCl | 36586.69 | 5 | 7317.338 | 8.812 | < 0.0001 |
| MgCl | 21865.94 | 3 | 7288.647 | 4.772 | < 0.01 |
| NHT | 56813.955 | 5 | 11362 | 11.342 | < 0.0001 |
| HQ | 52232.71 | 6 | 8705.452 | 7.379 | < 0.0001 |
| TA | 22772.595 | 3 | 7590.865 | 8.605 | < 0.0001 |

An ANCOVA analysis was again performed to test for statistically significant differences in increases in the percentage of unsuccessful egg-laying attempts between all chemicals. There was a highly significant statistical difference between all chemicals used in the analysis (ANCOVA, $F_{10, 275} = 28.9$, $p < 0.0001$) (Fig. 2.8). This again shows that the effectiveness of a chemical at successfully preventing egg-laying depended on the type of chemical which was, in turn, reflected by the concentration at which the chemical was effective at preventing egg-laying.

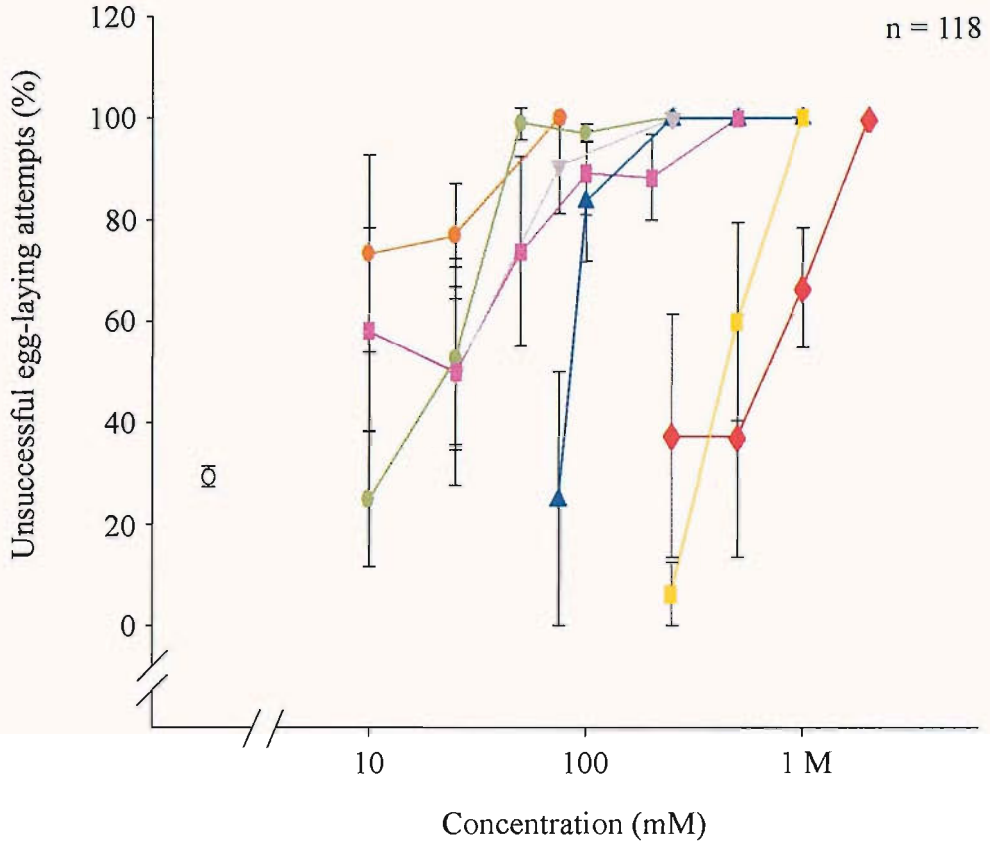



**Figure 2.8. Summary figure showing the effect of chemical type and concentration on the percentage of unsuccessful egg-laying attempts.** Data is shown for sucrose (red diamonds), LG (yellow squares), NaCl (blue triangles), MgCl (grey triangles), NHT (green circles), HQ (purple squares) and TA (brown circles). For each chemical the greater the concentration, the higher the percentage of unsuccessful egg-laying attempts. Individual points show means ± S.E.M. The data shown is based on 118 experiments each using 15 locusts.

### 2.3e The effect of chemical type and concentration on osmolarity and pH

The effect of chemical type and concentration on its osmolarity and pH was also analysed to determine whether pH and osmolarity of the substrate influenced egg-laying behaviour. As chemical concentration increased, a statistically significant increase in osmolarity was observed for all chemicals (Fig. 2.9).

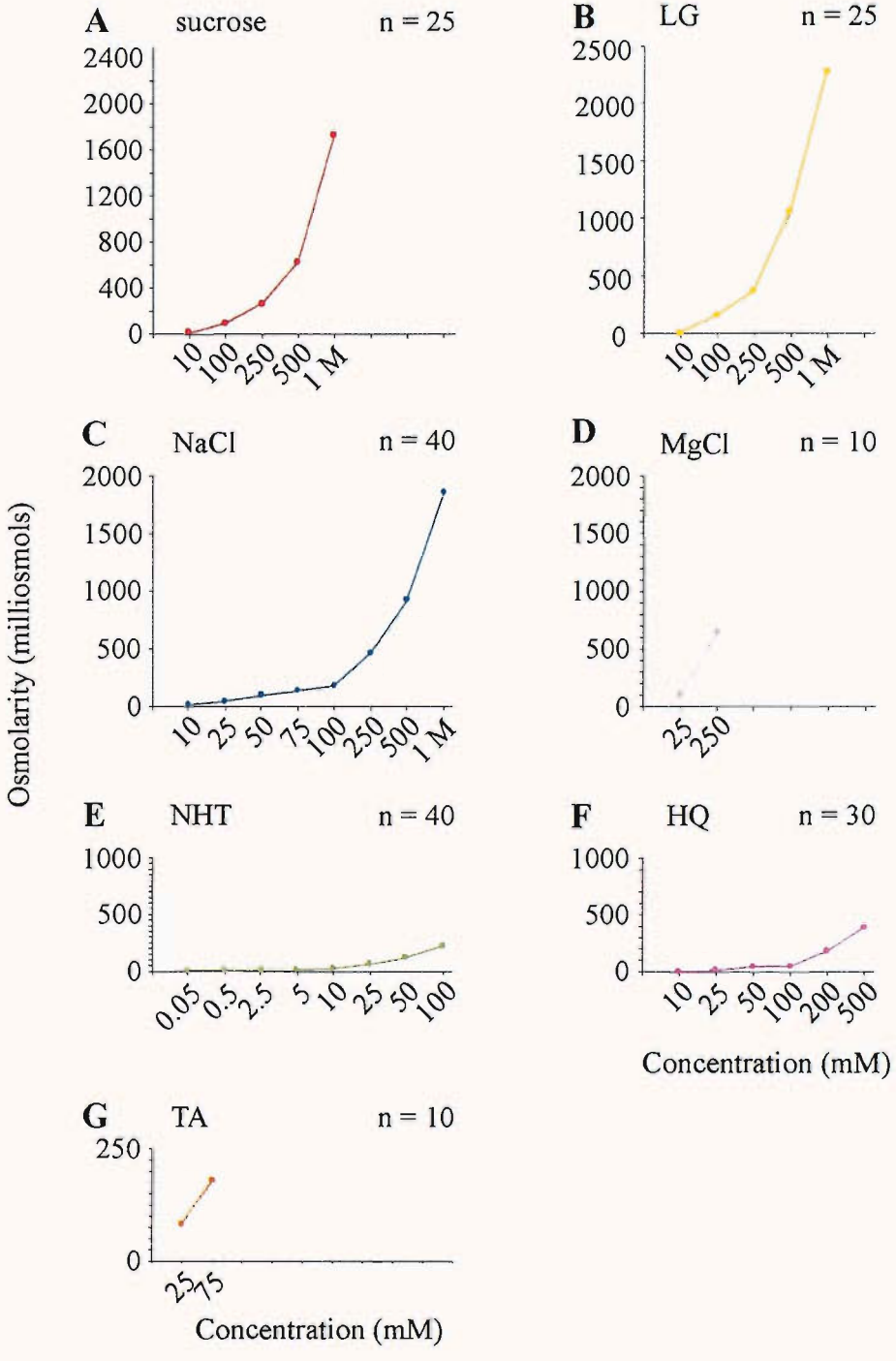



**Figure 2.9. The relationship between chemical concentration and osmolarity.** Data is shown for **A.** sucrose, **B.** LG, **C.** NaCl, **D.** MgCl, **E.** NHT, **F.** HQ and **G.** TA. Individual data points represent n = 5 measurements. Statistically significant increases in osmolarity ($p < 0.05$) were found with increasing chemical concentration for each chemical tested.

(sucrose, ANOVA, $F_{4, 19} = 60484.02$, $p < 0.0001$, LG, $F_{4, 20} = 16039.69$, $p <$

0.0001, NaCl, $F_{7, 32} = 83549.34$, $p < 0.0001$, MgCl, $F_{1, 8} = 105458.41$, $p < 0.0001$,

NHT, $F_{7, 32} = 31780.87$, $p < 0.0001$, HQ, $F_{5, 24} = 68974.35$, $p < 0.0001$ and TA, $F_{1, 8} = 24832.67$, $p < 0.0001$) (Table 5).

**Table 5: Analysis of variance of the relationship between chemical type and concentration and its osmolarity.** Increasing the concentration of all chemicals resulted in a statistically significant increase in osmolarity ($p < 0.01$).

| Chemical | Sum of squares | d.f. | Mean of squares | $F$ | $p$ |
|---|---|---|---|---|---|
| sucrose | 9549471.9 | 4 | 2387367.971 | 60484.021 | < 0.0001 |
| LG | 17274101 | 4 | 4318525 | 16039.686 | < 0.0001 |
| NaCl | 14233675 | 7 | 2033382.114 | 83549.342 | < 0.0001 |
| MgCl | 738208.9 | 1 | 738208.9 | 105458.41 | < 0.0001 |
| NHT | 208561.97 | 7 | 29794.568 | 31780.872 | < 0.0001 |
| HQ | 557542.67 | 5 | 111508.533 | 68974.351 | < 0.0001 |
| TA | 3724.9 | 1 | 3724.9 | 24832.667 | < 0.0001 |

Since the osmolarity of a chemical significantly increased with increasing chemical concentration, the possibility arose that the osmolarity was the main factor that affected egg-laying behaviour in locusts. A Pearson Product-Moment correlation analysis between the osmolarity of a chemical at various concentrations, and the concentration at which they increased the percentage of unsuccessful egg-laying attempts for all chemicals showed no correlation ($p > 0.05$) (Fig. 2.10A, B). This result shows that osmolarity was not primarily responsible for significantly increasing the percentage of unsuccessful egg-laying attempts.

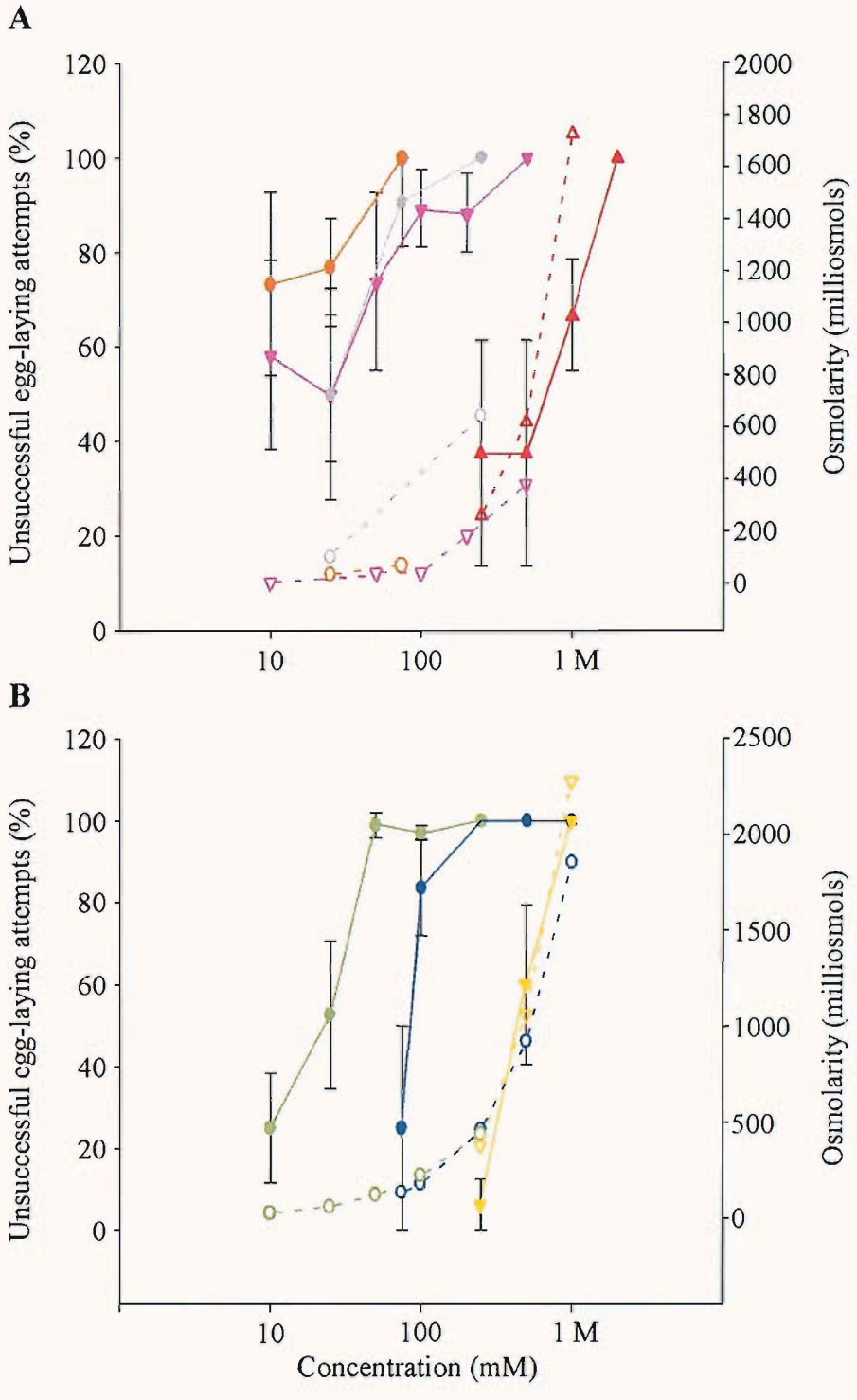



**Figure 2.10. The relationship between the osmolarity of chemical type and concentration and the percentage of unsuccessful egg-laying attempts.** Osmolarity data are denoted by dotted lines and open circles and triangles. **A.** Data is shown for TA (brown circles), MgCl (grey circles), HQ (purple triangles) and sucrose (red triangles). No significant correlation was found between the percentage of unsuccessful egg-laying attempts and osmolarity for all chemicals (Pearson Product-Moment correlation, $p > 0.05$). **B.** Data is shown for NHT (green circles), NaCl (blue circles) and LG (yellow triangles). No significant correlation was found between the percentage of unsuccessful egg-laying attempts and osmolarity for all chemicals ($p > 0.05$).

**Table 6: The effect of increasing chemical concentration on pH.** Increasing the concentration of all 4 dietary requirements resulted in no statistically significant variations in pH ($p > 0.05$). Increasing the concentration of the anti-feedants HQ and TA resulted in a statistically significant decrease in pH ($p < 0.01$). Although statistically significant differences in pH were observed between all concentrations of NHT, an overall concentration dependent decrease was not observed.

| Chemical | | | | | | | | $p$ |
|---|---|---|---|---|---|---|---|---|
| sucrose | Conc'n (mM) | 250 | 500 | 1 M | 2 M | | | |
| | pH | 6.38 ± 0.18 | 6.44 ± 0.18 | 6.75 ± 0.12 | 6.64 ± 0.16 | | | > 0.05 |
| LG | Conc'n (mM) | 250 | 500 | 1 M | | | | |
| | pH | 6.31 ± 0.02 | 6.27 ± 0.05 | 6.34 ± 0.04 | | | | > 0.05 |
| NaCl | Conc'n (mM) | 75 | 100 | 250 | 500 | 1 M | | |
| | pH | 5.70 ± 0.05 | 6.36 ± 0.23 | 5.84 ± 0.05 | 5.89 ± 0.02 | 6.05 ± 0.03 | | > 0.05 |
| MgCl | Conc'n (mM) | 25 | 75 | 250 | | | | |
| | pH | 5.70 ± 0.10 | 5.41 ± 0.081 | 5.66 ± 0.17 | | | | > 0.05 |
| NHT | Conc'n (mM) | 10 | 25 | 50 | 100 | 250 | | |
| | pH | 3.21 ± 0.003 | 3.49 ± 0.18 | 3.25 ± 0.08 | 3.26 ± 0.04 | 3.20 ± 0.10 | | < 0.01 |
| HQ | Conc'n (mM) | 10 | 25 | 50 | 100 | 200 | 500 | |
| | pH | 5.79 ± 0.06 | 5.12 ± 0.09 | 4.56 ± 0.21 | 4.64 ± 0.11 | 4.03 ± 0.10 | 3.80 ± 0.14 | < 0.01 |
| TA | Conc'n (mM) | 10 | 25 | 75 | | | | |
| | pH | 2.58 ± 0.02 | 2.57 ± 0.04 | 2.29 ± 0.06 | | | | < 0.01 |

The pH of chemicals that were dietary requirements was not dependent on chemical concentration (sucrose, ANOVA, $F_{5, 24} = 1.62$, $p > 0.05$, LG, $F_{4, 22} = 0.89$, $p > 0.05$, NaCl, $F_{7, 28} = 1.48$, $p > 0.05$, MgCl, $F_{2, 8} = 0.05$, $p > 0.05$) (Table 6, 7). By contrast, increasing the concentrations of all anti-feedants resulted in significant changes in pH (NHT, ANOVA, $F_{8, 30} = 9.82$, $p < 0.05$, HQ, $F_{5, 12} = 32.83$, $p < 0.05$ and TA, $F_{2, 8} = 138.45$, $p < 0.0001$) (Table 6). For example, at a concentration of 10mM, HQ had a pH of 5.79 ± 0.06, while at a concentration of 500mM, the pH of HQ decreased to 3.8 ± 0.14. Similarly, increasing concentrations of TA also decreased in pH with increasing chemical concentration. At its lowest concentration, TA (10mM) had a pH of 2.58 ± 0.02 and again decreased to a final pH of 2.29 ± 0.06. Although statistically significant differences in pH were observed between increasing concentrations of NHT, pH did not vary in a concentration dependent manner. For example, a 10mM concentration of NHT had a pH of 3.21 ± 0.003 compared with a 250mM concentration of NHT which had a pH of 3.2 ± 0.1 (Students T-test, $t = 0.067$, $p > 0.05$) (Table 6, 7).

**Table 7: Analysis of variance of the relationship between chemical type and concentration, and pH.** Four out of the 7 chemicals showed no statistical change in pH with increasing concentration (* $p > 0.05$). Statistically significant concentration dependent decreases in pH were observed for HQ and TA. Although statistically significant differences were found between the different concentrations of NHT, concentration dependent decreases were not observed.

| Chemical | Sum of squares | d.f. | Mean of squares | *F* | *p* |
|---|---|---|---|---|---|
| sucrose | 0.959 | 5 | 0.192 | 1.622 | > 0.05 |
| LG | 0.03306 | 4 | 0.008266 | 0.887 | > 0.05 |
| NaCl | 1.494 | 7 | 0.213 | 1.480 | > 0.05 |
| MgCl | 0.002758 | 2 | 0.001379 | 0.047 | > 0.05 |
| NHT | 4.366 | 8 | 0.546 | 9.815 | < 0.0001 |
| HQ | 7.915 | 5 | 1.583 | 32.833 | < 0.0001 |
| TA | 1.286 | 2 | 0.643 | 138.449 | < 0.0001 |

## 2.4 Discussion

It has been shown that female locusts can detect chemicals within a potential egg-laying substrate, prior to oviposition, and use that information to make a behavioural choice between suitable and unsuitable substrates for egg-laying. Moreover, it was shown that all chemicals act as egg-laying deterrents and inhibitors when applied at sufficiently high concentrations. Woodrow (1965) also showed that treating damp sand with various concentrations of anions, mono and divalent salts, also acted to prevent migratory locusts, *Locusta migratoria*, laying eggs when encountering chemicals at high concentrations. The work discussed here therefore extends the findings of Woodrow (1965) and reflects the results obtained from experiments that used chemicals representing all of the known taste classes over a range of concentrations, including salts (NaCl and MgCl), a carbohydrate (sucrose), an amino acid (LG), bitter tasting, toxic compounds (NHT and HQ) and a compound that represents a sour tasting quality (increases in $H^+$ ions - TA). This is the first time that such a wide range of chemicals, and their influence on the choice of a locusts egg-laying substrates, have been analysed.

### 2.4a Dependence of oviposition choice on substrate chemical composition

Many factors can influence an insect's choice of egg-laying site. The range and types of potential insect egg-laying sites are large and can include the undersides of leaves (Lepidopterans) (Simmonds, 2001; Rojas and Wyatt, 1999; Rojas *et al.* 2001), within the flesh of fruit (fruit flies) (Prokopy and Duan, 1998) or within soil or sandy substrates (Orthopterans) (Uvarov, 1977; Popov, 1980). To select appropriate egg-laying sites insects need to assess qualitative aspects of a substrate prior to egg-laying, including its surface characteristics, visual characteristics such as shape, and the presence or absence of certain chemicals (Hurter *et al.* 1999;

Simmonds, 2001; Jallow *et al.* 1999). All of these parameters can indicate the suitability of the substrate for egg-laying, which in turn may reflect the suitability of the substrate for offspring development (McCall and Cameron, 1995). In butterflies, the presence of specific chemicals (e.g. flavonoids and/or glucosinolates) on the leaves of a certain plant can indicate its identity and hence its suitability or unsuitability as a host for egg-laying (Simmonds, 2001). Similarly, the sweet potato whitefly, *Bemisia tabaci* actively discriminates against older host leaves when attempting egg-laying (Veenstra and Byrne, 1998). Moreover, as mentioned previously, the choice of an egg-laying site in insects usually reflects a food choice decision (Baur *et al.* 1998). The inability of an insect to make a behavioural choice between suitable and unsuitable egg-laying substrates would compromise the survival of the resultant offspring (McCall and Cameron, 1995).

In comparison to other insect families many Orthopterans, including locusts, also assess potential egg-laying substrates for qualities other than the presence of chemicals. The mechanical nature of the substrate for example, can indicate the suitability or unsuitability of a potential egg-laying substrate (Woodrow, 1965). Female sand crickets, *Gryllus firmus,* preferentially lay eggs at great depth in dry sand. Manipulation of the water content of sandy substrates influences the depth at which females lay their eggs. The lower the water content, the deeper *G. firmus* laid their eggs (Réale and Roff, 2002). Conversely, when the water content of a substrate was high, eggs were laid closer to the surface. Although the varying water content of the substrate would almost certainly influence its mechanical nature, and possibly affect the depth at which female *G. firmus* were able to lay its eggs, this could also be a strategy to avoid desiccation (Jorgensen and Rice, 1983). This also suggests that in a natural situation, should a potential egg-laying substrate for *G. firmus* have a high

water content (for example after rainfall) then eggs laid closer to the surface would be more susceptible to predation or parasitism.

Female desert locusts preferentially lay eggs within damp sand (Popov, 1980). This is to ensure rigidity of the excavated hole, preventing the walls of the hole collapsing on the eggs and also to prevent their desiccation (Jorgensen and Rice, 1983). The data presented within this chapter therefore reflect experiments that only manipulated the concentration of the chemicals used and show that the detection of chemicals within a substrate plays an integral part in the selection of a female locusts egg-laying site. Despite the ability to detect chemicals within a potential egg-laying substrate, it is unlikely that locusts would encounter the chemicals at the concentrations used in a natural context. In an ecological context however, this chapter does demonstrate that the locust's sense of taste could be exploited in future studies in an attempt at preventing egg-laying by gregarious locusts by using non-toxic, or mixtures of non-toxic chemicals.

### 2.4b The effects of chemical type and concentration on oviposition

Females laid egg pods in sand pots containing low concentrations of all chemicals tested, although greater numbers of egg pods were laid in control pots. This suggests that the presence of low concentrations of chemicals was still detected via taste receptors on the ovipositor valves, indicating that even at low concentrations a preference for control pots prevailed. Conversely, the highest concentrations of all chemicals tested stopped egg-laying in chemically treated pots. Six out of the seven chemicals used in the analysis, resulted in a concentration dependent reduction in the number of egg pods laid in the test pots although the exception; 2 M sucrose, stopped egg-laying, a statistically significant reduction in egg pods laid was not observed.

Surprisingly, despite preventing egg-laying, high concentrations of the chemicals tested did not always prevent the digging of substrates. This extends the

work performed by Woodrow (1965), who immersed severed abdomen producing digging movements of the ovipositor valves, into a 2 M solution of NaCl. Although a rejection response was initially observed, the digging rhythm eventually returned to normal despite being submerged in the 2 M solution of NaCl. This suggests that the contact chemosensory system of the female locust may have the capability to adapt to otherwise aversive concentrations of a chemical, perhaps at the level of the sensory neurons themselves (Newland, 1998). It was also clear that there was a concentration-dependent effect of a chemical on the duration for which female locusts exhibited digging. Locusts, however, were observed to dig substrates containing high concentrations of a given chemical. This suggests that locusts will sample and attempt to lay eggs within a sub-standard substrate should an ideal substrate not be available. This again extends the findings of Woodrow (1965) that, to a certain extent, the chemosensory system of the locust can be overridden, depending on the current state of the external environment i.e. the lack of an ideal egg-laying substrate. Whether this occurs at the level of the peripheral nervous system of the locust (the sensory neurons of the basiconic sensilla) or the central nervous system, is at present unknown.

### 2.4c The role of pH and osmolarity in the egg-laying behaviour of locusts

To rule out any potential effects of pH or osmolarity on the egg laying behaviour of locusts measurements of osmolarity and pH were made on all chemicals across the entire range of concentrations used. For pH, there were no statistically significant differences between different concentrations of chemicals that represented dietary requirements. Conversely, anti-feedants all showed a statistically significant reduction in pH with increasing concentration. The effectiveness of a chemical at preventing egg-laying cannot, however, be primarily attributed to pH. For example,

NaCl, whose pH did not vary with increasing concentration, was effective at preventing egg-laying, as was TA, where increasing concentrations of TA resulted in a decrease in pH. Moreover, NaCl was more effective at preventing egg-laying than HQ, whose pH again decreased with increasing concentration. Although the sour taste quality has been shown to rely on the detection of pH (Lyall *et al.* 2001), other taste qualities such as bitter are not dependent on the detection of pH at the level of the taste receptor but instead rely on G-proteins such as gustducin (Akabas *et al.* 1988). This contradicts the findings of this study, as both representatives of bitter tasting, toxic chemicals (NHT and HQ), were shown to decrease in pH with increasing concentration. Moreover, the behavioural responses to all chemicals e.g. the significant reduction in egg pod number, cannot be explained by pH alone as increases in the concentration of dietary requirements (sucrose, LG, NaCl and MgCl) did not result in any variation in pH. Thus, pH alone is not the major determinant in the locust's response to the chemical composition of the substrate.

Increasing concentrations of all chemicals also resulted in statistically significant changes in osmolarity. It is therefore tempting to attribute behavioural responses to a chemical, not only to the presence of the chemical itself, but also to a secondary effect of osmolarity. However, a Pearson Product – Moment correlation analysis between the osmolarity of a chemical and the percentage of unsuccessful egg-laying attempts showed no correlation. As with pH, osmolarity alone does not explain behavioural responses to the chemical composition of an egg-laying substrate. The effects of osmolarity on taste responses have, however, been reported in vertebrates. When isolated rat TRC's were stimulated with 150mM NaCl, action potentials were subsequently recorded in the CT nerve (Lyall *et al.* 1999). Increasing the osmolarity by adding 300mM of mannitol or cellobiose to the stimulus solutions increased the amplitude of compound action potentials recorded from the CT nerve.

The increased responses are thought to be caused by a decrease in volume of the TRC as a result of increasing osmolarity. Although a change in TRC volume as a result of increases in osmolarity may affect transduction events of contact chemical stimuli (Gilbertson, 2002), behavioural responses to osmolarity have not been reported, and were not evident in this study.

In summary, the highest concentrations of all of the chemicals used prevented egg-laying, although the digging of egg-laying substrates was not completely prevented. Increasing the chemical concentration within the test pots resulted in concentration dependent decreases in the duration spent exhibiting egg-laying behaviour. No consistent concentration dependent effects were observed on the number of egg-laying attempts, although with increasing chemical concentration the percentage of these egg-laying attempts became increasingly unsuccessful. The concentration at which the chemicals used in the analysis acted aversively depended on the chemicals identity. This is shown in the summary figures 2.3, 2.5 and 2.8. The data points that represent known anti-feedants tend to act aversively at lower concentrations than chemicals that represent dietary requirements (sucrose, LG, NaCl and MgCl). Consequently, the data points of these chemicals are located to the left-hand side of the summary figures. As a chemical becomes nutritionally more relevant (carbohydrates, salts and amino acids), the data points of these chemicals shift further to the right hand side of the graph. The only exception with all three summary figures are the results for HQ, the data points for which show a degree of overlap with the data points that represent values for the other 6 chemicals. This data closely parallels those of Rogers and Newland (2000), who showed a similar relationship between chemical type and the concentration ranges over which they became aversive. Again, the anti-feedant NHT was situated to the far left hand side of the graph with the data points for NaCl, LG and sucrose situated with increasing

distance away from the data points of NHT respectively. All chemicals used in the analysis had the potential to become aversive and it appears that the detection of aversive quality is of fundamental importance to the locust, and not the identity of the chemical.

# Chapter 3 – Anatomy and physiology of the locusts abdominal digging movements

Chapter 3

# Anatomy and physiology of the locusts abdominal digging movements

## 3.1 Introduction

Rhythmic movements are common in all animals and underlie vital physiological and behavioural processes such as the heartbeat, gut movements, mastication, ventilation, locomotion and copulation (see review by Selverston *et al.* 1997). Rhythmic movements are often the subject of physiological and behavioural investigation as they are easy to elicit and provide many repetitions of the same sequence of behaviour. This also permits a detailed analysis of the patterns of neural connections, synaptic properties and the effects of neuromodulators and neurotransmitters on behaviour. The investigation of such rhythms may therefore reveal aspects of rhythm production and maintenance that are common to all rhythmic behaviour.

Neural networks that produce rhythms without sensory input from the peripheral nervous system are termed CPG's (Brown, 1911), and these underlie several forms of rhythmic movement. Numerous studies of vertebrates and invertebrates have shown that rhythmic behaviours can be produced and maintained in isolated central nervous systems despite the absence of sensory input (Delcomyn, 1980). Typical models for CPG research include the neuronal networks underlying locomotion in the lamprey (Hu *et al.* 2002; Zhang and Grillner, 2000; Deliagina *et al.* 2000), turtle (Earhart and Stein, 2000) and decerebrate cats (Beloozerova and Rossignol, 1999; Misiaszek and Pearson, 1997; Douglas *et al.* 1993). In invertebrates the most thoroughly researched examples of CPG's include those controlling locomotion in stick insects (Brunn, 1998) and *Caenorhabditis elegans*

(Hardaker *et al.* 2001), flight in locusts (Wilson, 1961), the feeding movements of molluscs (Elliott and Benjamin, 1985; Murphy, 2001; Staras *et al.* 1998) and control of the stomatogastric rhythms of crustaceans (Simmers *et al.* 1995b).

Rhythmic motor patterns usually have different, clearly defined phases, during which movements are produced by different groups of muscles that are innervated by their respective groups of motor neurons (Cropper and Weiss, 1996). Although some motor neurons are endogenously rhythmic (Knop *et al.* 2001), the different phases and timing of a motor pattern can be separated and co-ordinated by different types of interneurons. The reliable production and maintenance of a motor pattern and its resultant behaviour therefore depends on the synaptic connectivity between the different groups of motor neurons and between various interneurons and motor neurons (Staras *et al.* 1998).

### 3.1a CPG's and oviposition

In insects, rhythmic movements are also involved in oviposition (Woodrow, 1965) in which specialised structures located at the distal-most segment of the abdomen, called the ovipositor, function to lay eggs on or within a suitable substrate. Not all insect species possess true ovipositors, the anatomy of which can vary considerably across the insect class (Chapman, 1969). Structures range from elaborate valve complexes, to simple, needle-like structures designed to impale and place eggs within a substrate or another animal (Chapman, 1969). The rhythmic movements of the ovipositor are usually produced by direct, CPG-innervated muscular attachments to the ovipositor itself, or indirectly by rhythmic movements of the entire abdomen (Facciponte and Lange, 1996; Vilhelmsen *et al.* 2001). Both types of rhythm serve the purpose of manoeuvring the abdomen into position to place eggs on or within a suitable substrate.

Thompson (1986a) showed that isolating the abdomen from the thorax of locusts could reliably elicit rhythmic movements of the ovipositor. The digging movements of the valves have three different phases (a tri-phasic motor pattern) produced by four groups of muscles; the protractors, the openers, the closers and the retractors (Thompson, 1986a). The protractor muscles drive the valves posteriorly out of the abdomen to push the valves into an egg-laying substrate. This is then followed by the opener muscles swinging the valves open to displace the substrate dorso-ventrally. Lastly, the valves simultaneously close and retract in preparation for another cycle of digging (Thompson, 1986a). The oviposition digging rhythm also alternates intermittently with a "tamping rhythm," which is physiologically characterized by activity in the closer muscles alone. These movements function to smooth the sides of the excavated hole to prevent the hole from collapsing during digging (Thompson, 1986a; Seymour, 1990).

Although the digging rhythm has been extensively studied (Thompson, 1986a), long-term recordings of the opener muscles have not been described. This chapter details the anatomy of the female locust's abdomen and ovipositor valves. This chapter also assesses the use of the isolated abdomen for use in contact-chemosensory related behavioural experiments.

## 3.2 Materials and Methods

### 3.2a Locusts

Female desert locusts, *Schistocerca gregaria* (Forskål) maintained and reared as described in *Chapter 2*.

### 3.2b Anatomical methods

The dissection of isolated abdomen for anatomical, behavioural and physiological study began by decapitation of the locust using a twisting motion followed by a sharp pull of the head, which served to remove the gut. The structure of the ovipositor valves and cuticular apodeme were determined by removing the valves and apodeme from the abdomen. All attached musculature was removed using a scalpel. The remaining musculature was removed by placing it in 100% alcohol overnight rendering the remaining musculature brittle and easy to remove. The isolated abdomen was photographed using a Nikon coolpix 950 digital camera fitted to the trinocular head of a Zeiss Stemi 2000-CS dissecting microscope.

Detailed drawings of the muscles involved in the rhythmic digging movements of the ovipositor valves were made by pinning open the abdomen using minuten pins in a Sylgard-lined dish containing saline. A superficial mid-ventral or mid-dorsal incision of the abdomen was made to expose the muscles. The spermatheca and oviducts were removed from ventrally dissected preparations to allow access, while in dorsal preparations the remaining gut and its contents were removed. Ovipositor muscles were not damaged during either procedure.

The precise locations of muscles, their insertions and attachments to both the cuticle and ovipositor valves were determined using the descriptions of Albrecht (1953), Thompson (1986a) and Seymour (1990). For the precise identification of

muscles, dissected preparations were then stained with Toluidine blue. Drawings were made with the aid of a Zeiss Stemi 2000 CS binocular microscope.

### 3.2c Physiological methods

Muscle activity was recorded from three of the four main muscle groups involved in digging, the ventral opener, closer and retractor muscles. Recordings were obtained using pairs of 63 μm copper wire, insulated except for their tips, pushed through small holes in the cuticle into the muscles under study. Myogram wires were then secured in place using cyanoacrylate glue. After each experiment the animal was dissected and the locations of the wires visually confirmed.

Signals from the electrodes were amplified with an AC pre-amplifier and displayed on a Tektronix TDS 210 oscilloscope, digitised using a Cambridge Electronic Design 1401 interface and displayed and analysed using Spike 2 v4.0 software. Experiments were based on results from 34 locusts.

## 3.3 Results

### 3.3a External anatomy of the female locust abdomen

The abdomen of a female locust consists of 11 dorsal tergites and 8 ventral sternites (Fig. 3.1A, B) (Albrecht, 1953).

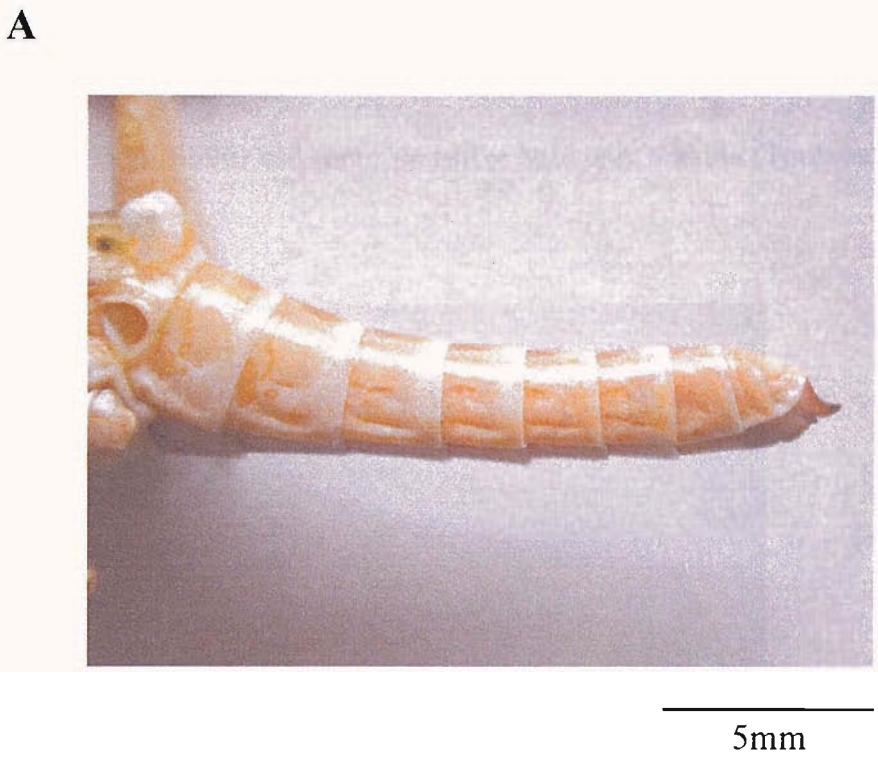



**Figure 3.1. The anatomy of the female locust abdomen. A.** A photograph showing the lateral view of an abdomen. **B.** A drawing showing the lateral view of an abdomen. The dorsal section of an abdomen consists of 11 separate tergites (Tg). In contrast, the ventral section of the abdomen consists of 8 separate sternites (St). The dorsal ovipositor valves project from tergite 11 and the ventral valves from sternite 8.

At the tip of the abdomen are a pair of highly sclerotised cuticular appendages, the ovipositor valves, which consist of a pair of dorsal and a pair of ventral valves. The dorsal valves project from tergite 11 and can be further divided into an epiproct and a paraproct (Fig. 3.2). The ventral valves extend beyond sternite 8, the sub-genital plate (Fig. 3.2A, B). A small pair of cerci are also present on tergite 11, which are covered with mechanosensitive hairs that respond to physical deflection (Kalogianni, 1996) and chemosensitive basiconic sensilla (Tousson and Hustert, 2000).

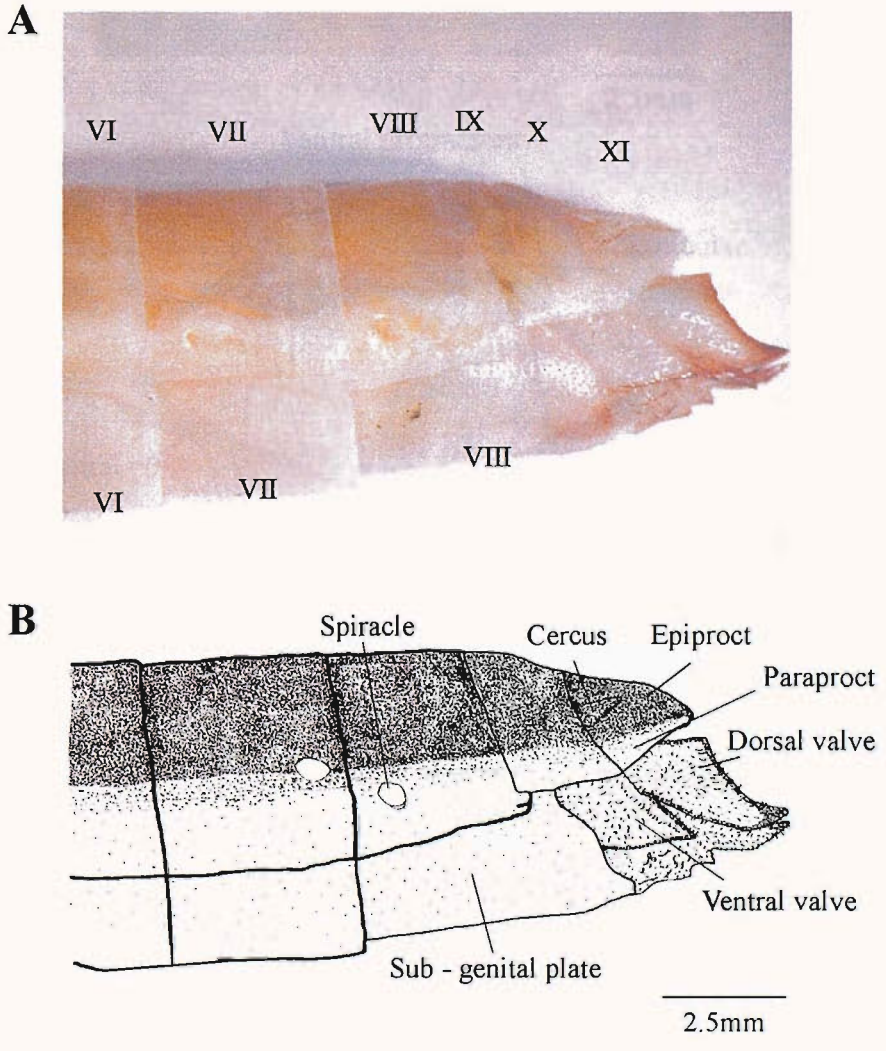



**Figure 3.2. Detailed anatomy of the distal end of the female abdomen. A.** A lateral view of the abdomen and the ovipositor valves. Tergites 6-11 (dorsal) and sternites 6-8 (ventral) contain the muscles that produce the digging movements of the valves. **B.** Two pairs of valves are involved in the digging of substrates, the dorsal and ventral ovipositor valves. The dorsal valves project from tergite 11, which consists of a dorsal epiproct and paraproct region. The ventral valves project distally from sub-genital plate (sternite 8). A mechanosensitive cercus and a spiracle, a

structure involved in ventilation are also labelled. At least 1 spiracle is found on each tergite of the abdomen, normally laterally near the midline of the animal (Albrecht, 1953).

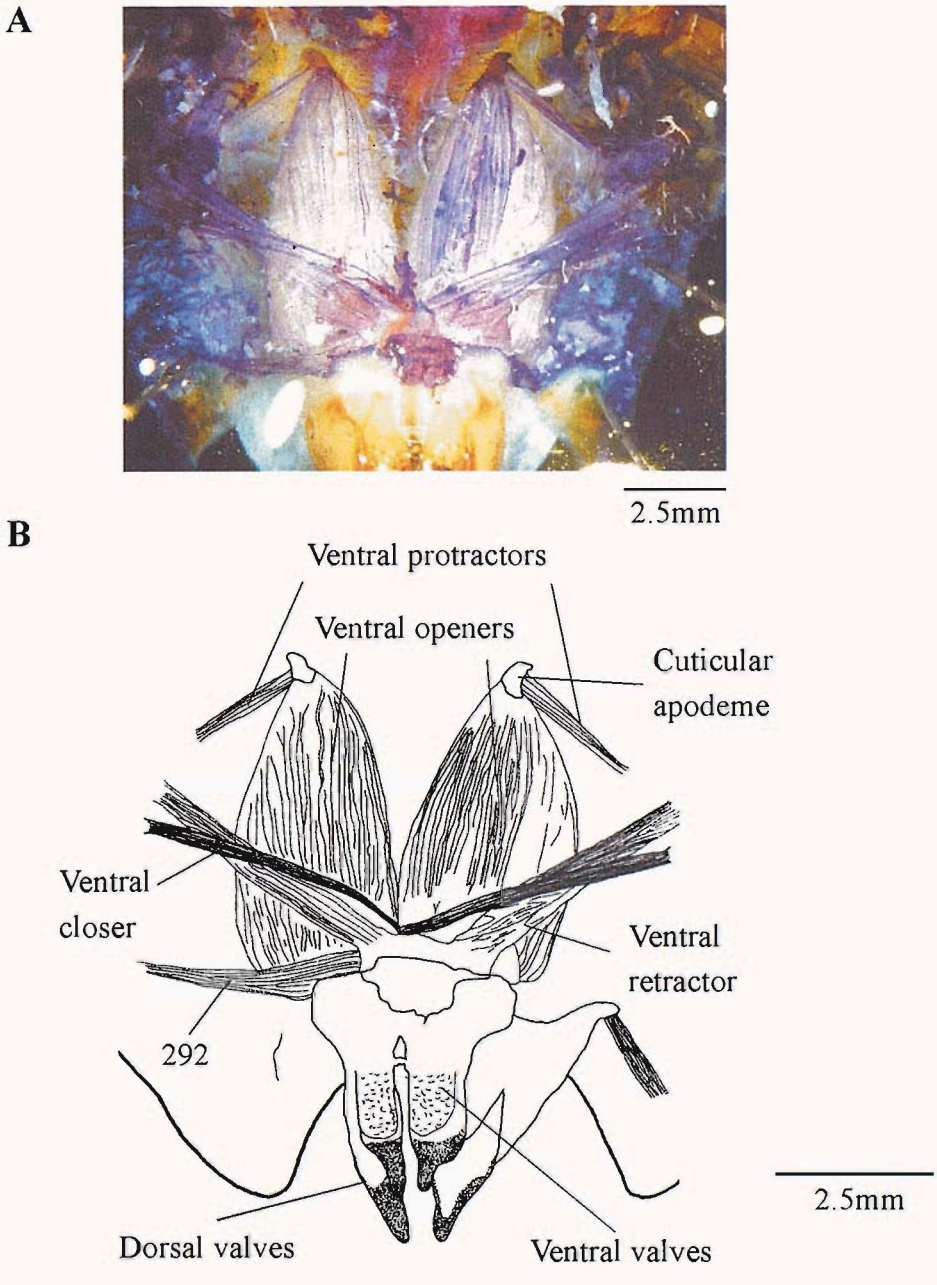



**Figure 3.3. The musculature of the ventral ovipositor valves. A.** Sternites 6-8 were dissected using a ventral midline incision of the abdomen. The muscles are stained with toluidine blue for ease of identification. **B.** A drawing of the ventral valve musculature. Four pairs of muscles are involved in the movements of the ventral ovipositor valves; the ventral protractors, openers, closers and retractors. Also labelled is muscle 292 which attaches to the base of the ventral ovipositor valve (Albrecht, 1953). This muscle is not directly involved in the digging movements of

the valves. An unidentified muscle attaches to the anterior most process of the dorsal ovipositor valves.

### 3.3b Functional anatomy of the valve musculature

The musculature that produces the movements of the ovipositor valves consists of four pairs of muscles that insert on both ventral and dorsal regions of the abdomen. All muscles are situated between the posterior-most midline of tergite 6 and sternite 6 and extend to tergite 11 and sternite 8 respectively, where they attach to various regions of their respective ovipositor valves.

#### Ventral valve musculature

The ventral protractor muscle is a very short, thin band of muscle whose insertion point is at the anterior tip of a rigid cuticular apodeme (Fig. 3.3A, B). This muscle stretches ventro-laterally to attach onto the inner cuticle of the mid-dorsal region of tergite 7. The contraction of this muscle pushes the cuticular apodeme (and therefore all of the musculature attached to the apodeme) out of the abdomen and therefore contributes to the first stage of digging.

The ventral opener muscles are the largest of the ventral valve muscles. The insertion points for both ventral opener muscles occur exclusively along the entire length of the cuticular apodeme. The muscles themselves make a direct attachment to the ventral valves via membrane. When the ventral opener muscles contract the valves open to displace the substrate and advance subsequent digging movements (Thompson, 1986a).

The ventral closer muscles are very thin bands of muscle. These muscles attach onto the inner surface of the posterior-most area of the cuticular apodeme and function to pull the valves shut. The ventral closer muscles attach to the ventral surface of sternite 7, with left and right closers attaching to the left and right of the

ventral mid-line. The ventral retractor muscles are larger bands of muscle compared to the ventral closers. These muscles insert directly onto the ventral valves via a thin band of rigid cuticle and project anterio-dorsally to attach to the inner surface of tergite 7, just above the midline of the abdomen (Fig. 3.3A, B; Fig. 3.5A, B).

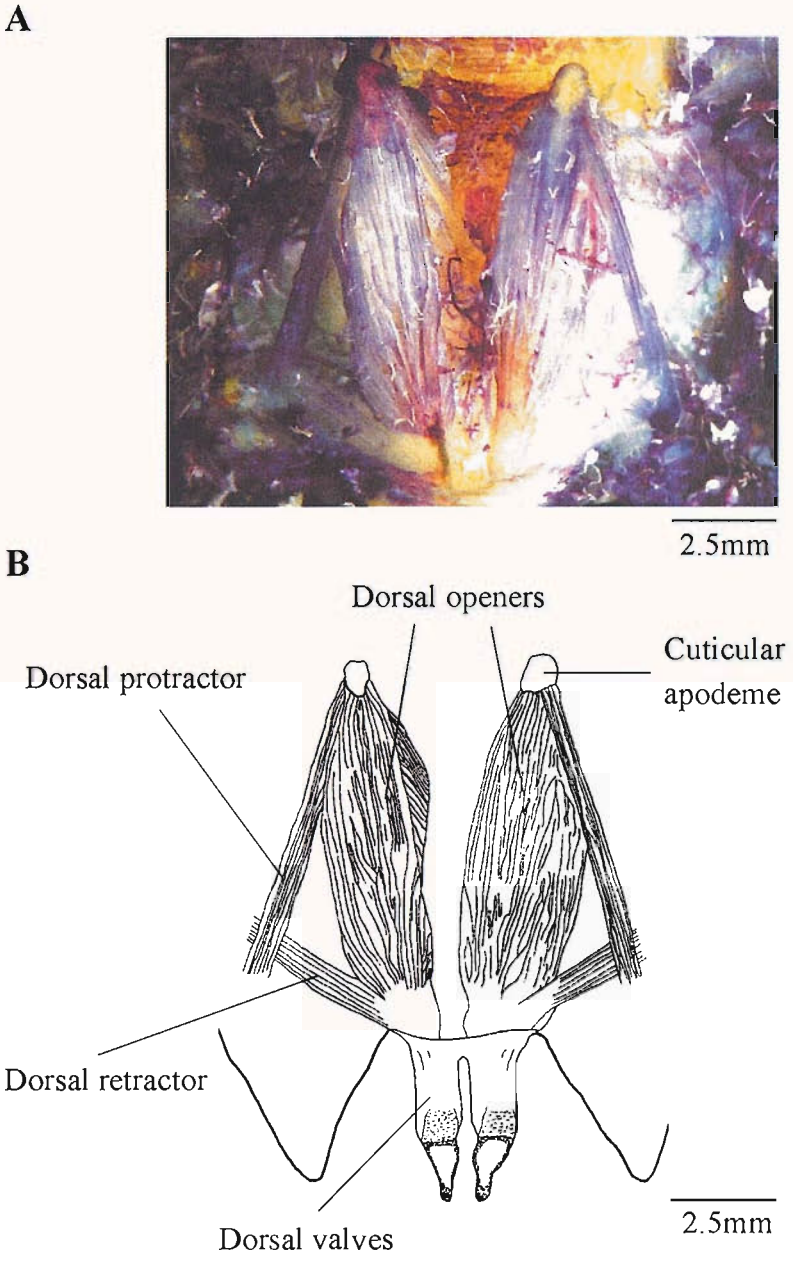



**Figure 3.4. The muscles of the dorsal ovipositor valves. A.** A dorsal dissection of tergites 8-11. The dissection was made by making an incision in the dorsal midline of the locust and pinning the cuticle open using minuten pins. Muscles were then stained using toluidine blue. **B.** A drawing of the musculature involved in the cyclical movements of the dorsal valves. The muscles involved are 3 large pairs of protractor, opener and retractor muscles. Dorsal closer muscles are present, but are much reduced compared to their ventral counterparts and not visible in the photograph.

**Dorsal valve musculature**

The dorsal region of tergites 6 to 10 contains the muscles that move the dorsal ovipositor valves. The arrangements of the muscle groups show some similarities to the ventral valve musculature, but also show some distinct anatomical differences. The dorsal valves are also moved by four groups of muscles that produce the digging movements. In contrast to the ventral closer muscles, the dorsal closer muscles are much reduced in size (not visible in Fig. 3.4A). In addition, the dorsal protractor muscles are elongate compared to the much shortened ventral protractor muscles (Fig. 3.4A, B).

The dorsal protractors, like the ventral protractors, are very thin bands of muscle that attach to the anterior most tip of the cuticular apodeme and project postero-dorsally to attach to the inner surface of tergite 10. They function in the same manner as the ventral protractor, to push the dorsal valves out of the abdomen by pulling the cuticular apodeme backwards. The dorsal opener muscles are very similar in size and shape to the ventral opener muscles and are the largest of the dorsal valve musculature. In comparison to the ventral opener muscles, these muscles do not make any attachments to the cuticle and are attached directly to the cuticular apodeme. When the dorsal opener muscles contract, the dorsal ovipositor valves swing open and assist the ventral openers in displacing the substrate.

The dorsal retractor muscles are thin bands of muscle that attach to a tendon bridging the gap between the dorsal opener muscles and the dorsal valves. These muscles project dorso-laterally to attach onto the inside cuticle of tergite 8 and function to pull the dorsal valves back into the abdomen on completion of the digging movements (Fig. 3.4A, B; Fig. 3.5A, B).

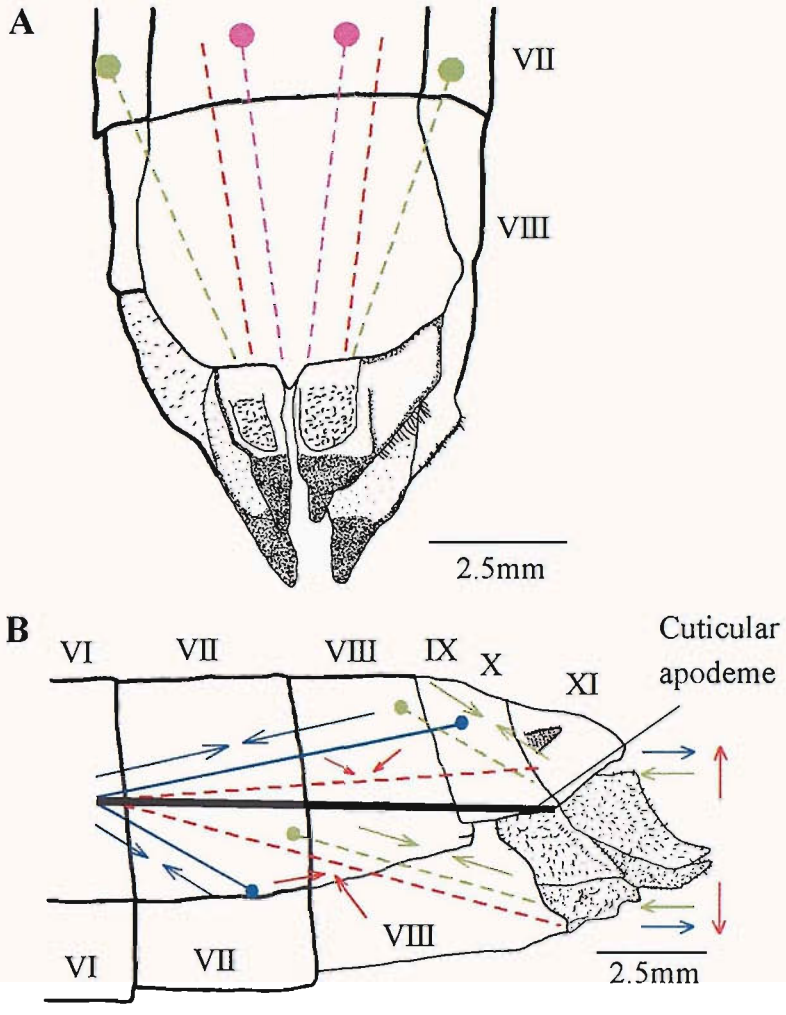



**Figure 3.5. Ventral and lateral views of the abdomen showing muscle insertions and their lines of action. A.** Red dashes represent the ventral opener muscles that project from the valves to attach onto a cuticular apodeme (see Fig. 3.6A, B and C), the tips of which are located in tergite 7. Pink dashes represent the closer muscles which attach to the inner surface of sternite 7 (closed circles). Green dashes represent the ventral retractors line of action that project antero-laterally from the base of the ventral valves and attach laterally to the cuticle of tergite 7. **B.** Solid blue lines represent the lines of action of the dorsal and ventral protractors which project dorso- and ventro-laterally, respectively from the cuticular apodeme. Dorsal protractors attach to tergite 10 and the ventral protractors attach to tergite 7 just above the midline. Dorsal retractors project dorso-laterally to attach to tergite 8. Red dashes represent the lines of action of the dorsal and ventral opener muscles which have no attachments on the cuticle but are attached primarily to the cuticular apodeme. Coloured arrows adjacent to the lines of muscle action represent the direction of muscle contraction. Arrows located to the right of the ovipositor valves represent the direction in which the ovipositor valves move as a result of muscle contraction.

The lines of action of the identified muscle groups are described diagrammatically in Fig. 3.5A and B. Figure 3.5A shows the arrangement and lines of action of the ventral opener (red dashes), the ventral closer (pink dashes) and the

ventral retractor muscles (green dashes). Filled circles detail the insertion points of these muscles onto the inner surface of the cuticle.

Figure 3.5B shows a lateral view of the abdomen detailing the lines of action of the protractor, opener and retractor muscles. The solid horizontal line represents the cuticular apodeme (see Figure 3.6 and 3.7), with which all muscles involved in digging attach. Red dashes again represent the position of the dorsal and ventral openers. Green dashes show the position of the dorsal and ventral retractors. The solid blue lines that project diagonally from the tip of the cuticular apodeme to sternite 7 and tergite 10, represent the positions of the protractor muscles. Filled circles represent the locations of muscle insertion onto the inner surface of the cuticle.

### 3.3c The apodeme-ovipositor valve complex

The apodemes consist of two blades of rigid cuticle that serve as attachment points for dorsal and ventral protractor and opener muscles. Both apodemes articulate with the dorsal and ventral valves and allow the opening and closing of the valves. When intact, the apodemes directly connect with both the dorsal and ventral ovipositor valves (Fig. 3.6A). The various components of the apodeme-valve complex were identified by dehydrating the intact valve-apodeme complex for 12 hr in 100% ethanol. This rendered any remaining membrane, musculature or tendon brittle and therefore allowed its removal.

The dorsal valves are highly sclerotised rigid structures that articulate with the posterior-most end of the cuticular apodemes via 1 ($R_1$) of 2 dorsal ridges. Dorsal ridge 2 ($R_2$) serves as an attachment point of the ventral closer muscles, which when activated pull the dorsal valves shut. Conversely, the ventral ovipositor valve is made up of three plates termed the "intervalvular plates" (Iv 1-3). The ventral

ovipositor valve is a much-reduced structure compared to the dorsal ovipositor valve and consists of a digging portion (the ventral valve) that connects with 3 intervalvular plates. The intact ventral valve articulates with the posterior-most tip of the cuticular apodeme via two small sclerites. This allows the opening and closing of the ovipositor valves during digging (Fig. 3.6B) (Seymour, 1990).

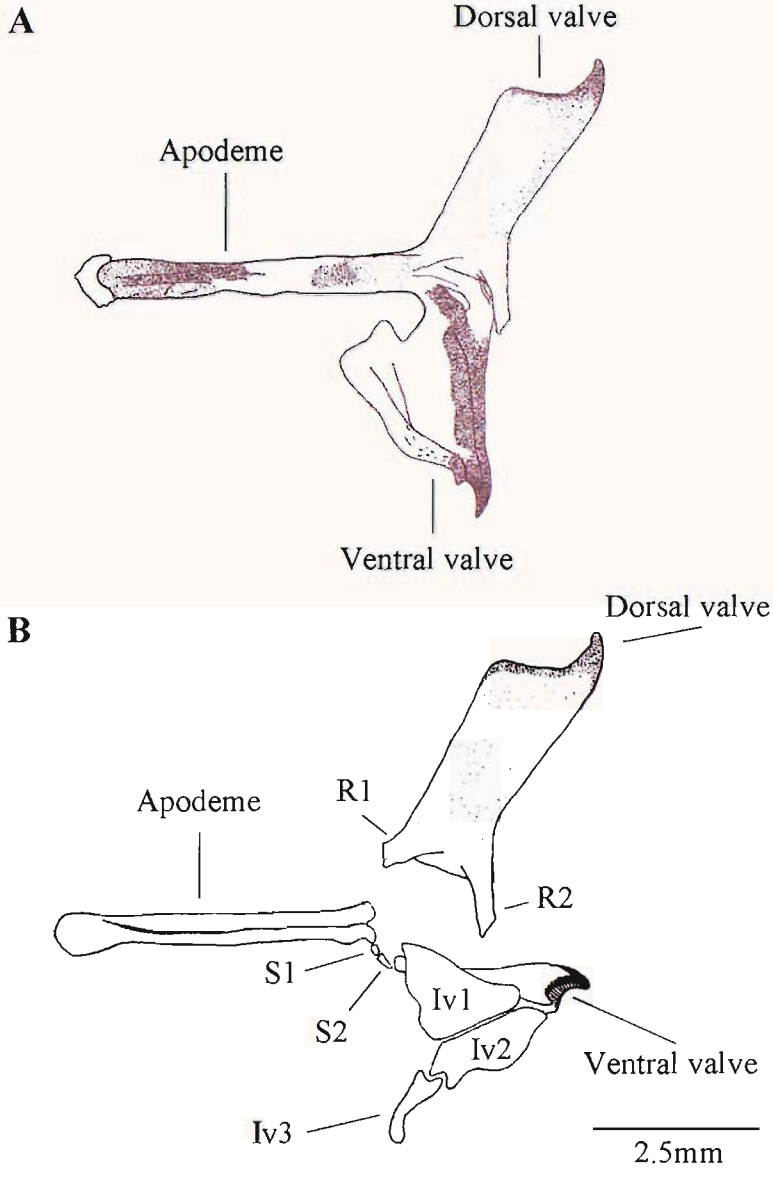



**Figure 3.6. The cuticular apodemes of the ovipositor valves. A.** Lateral view showing a blade-like cuticular apodeme and the dorsal and ventral valves. **B.** Lateral view showing the individual components of the cuticular apodeme-ovipositor valve complex. In contrast to the dorsal valves, the ventral valves are made up of 3 intervalvular plates (Iv 1-3) that are connected by membrane in an intact animal. Connections of the apodeme to the dorsal valves are by 1 of 2 dorsal ridges (R1) via membrane. R2 serves as an attachment point for the ventral closer muscles. In the case of the ventral valves, connection is made by 2 small sclerites (also via membrane) which allow the articulation of the ventral valves when opening and closing.

A dorsal view of the ovipositor valve-apodeme complex shows that it consists of two apodemes. This allows the attachment of all four protractor and opener muscles (Fig. 3.7).

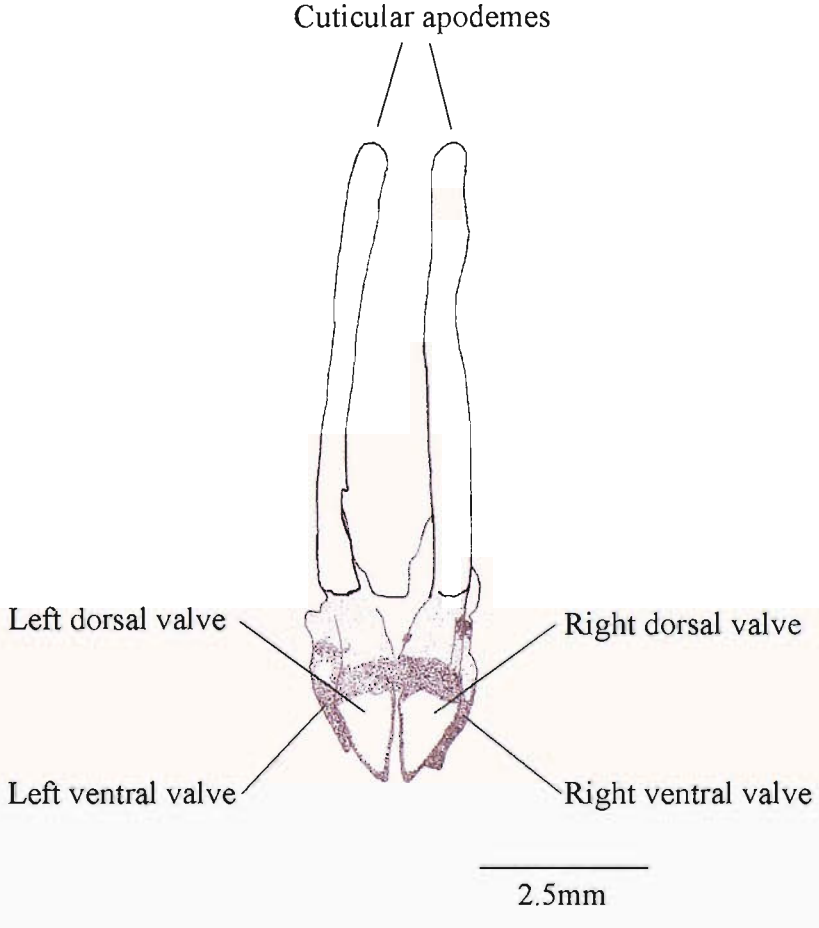



**Figure 3.7. A dorsal view of the ovipositor valves and cuticular apodemes.** The apodemes are blade-like in appearance and project anteriorly. The apodemes serve as the primary articulation points for the dorsal and ventral opener muscles which, when active, swing both the dorsal and ventral valves open. This movement functions to remove sand from the excavated hole and allows the progression of digging.

### 3.3d The digging movements of the ovipositor valves

The ovipositor muscle groups are activated in a distinct pattern and show distinct phase relationships (Thompson, 1986a; Seymour, 1990). During a cyclical digging movement the valves are protracted distally out of the abdomen (Fig. 3.8B).

The ovipositor valves then begin to swing open (Fig. 3.8C) until they are fully opened (Fig. 3.8D). The opening movement is then followed by a simultaneous closing and retraction movement of the ovipositor valves (Fig. 3.8E-F) until they return to their resting closed position (Fig. 3.8A). This pattern of movement is then repeated in order to advance the abdomen and continue the excavation of the substrate.

### 3.3e Activity patterns of the muscles involved in the digging rhythm

The activity patterns of all four muscle groups and their phase relationships have been well characterized by Thompson (1986a, b) and Seymour (1990). The locust oviposition digging rhythm has been shown to be tri-phasic (Thompson 1986a), during which the protractor muscles burst slightly before, and during, the opener muscle bursts. Protractor and opener muscle activity ceases and is followed by simultaneous closer and retractor muscle activity, mirroring the behavioural descriptions of the valve movement above. The size and thinness of the protractor muscles rendered recordings difficult to obtain. Recordings of only three out of the four muscle groups have therefore been included here. The relationships and variation between bursting of all four opener muscles have, however, not been previously described. Long-term recordings of opener muscle activity patterns were also performed and analysed, to assess the suitability of the preparation for further investigation into the behavioural responses of the ovipositor valves to different chemicals.

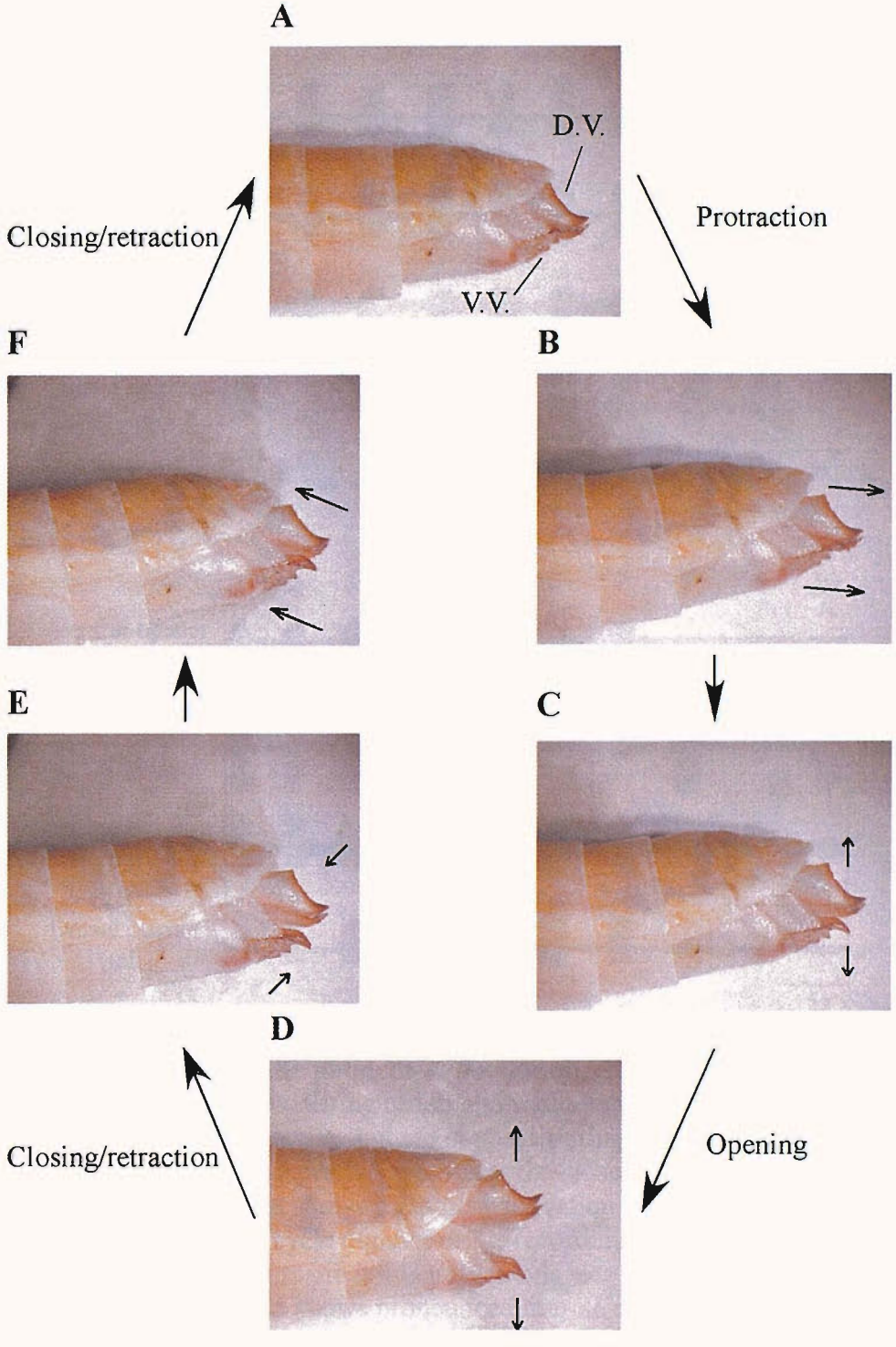



**Figure 3.8. Photographs showing the cyclical movements of the locust ovipositor valves. A.** The dorsal valves (D.V.) and ventral valves (V.V.) in their closed position. **B.** The protractive movements of the ovipositor push the valves out of the abdomen. **C-D.** The opening movements of the ovipositor valves (D=fully open). **E-F.** The closing and retraction movements of the valves, returning the valves back to their original closed position.

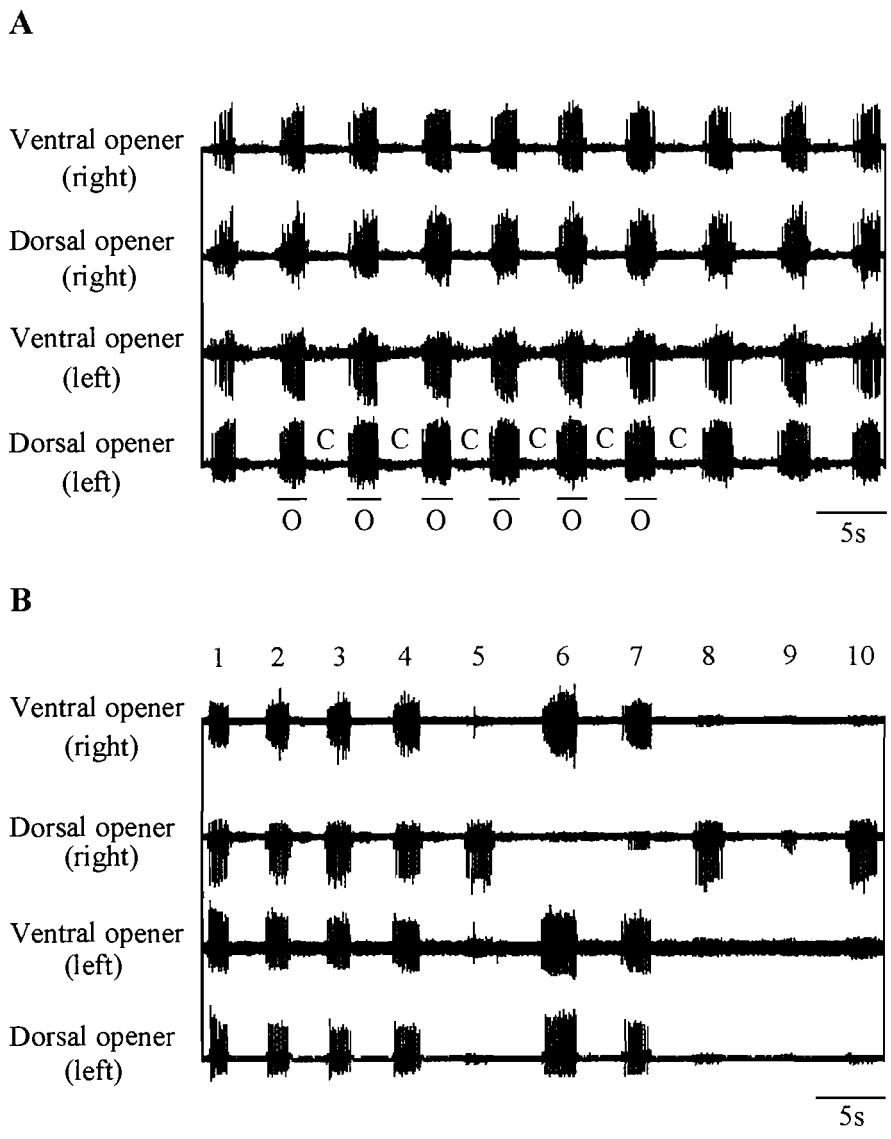



**Figure 3.9. Variations in the patterns of opener muscle activity. A.** A recording from all 4 opener muscles consisting of left and right dorsal and ventral opener muscles. All 4 muscles are active and are in phase during the opening (O) movement of the digging rhythm. The letter 'C' represents the closed phase of the digging rhythm. **B.** A recording that shows reciprocal activation of the opener muscles. The numbers 1-10 represent the cycle number of the recording. Note that after opener burst 4, muscle activity occurs predominantly in one side of the animals' ovipositor valves. For example, cycle 5 shows pronounced muscle activity in the right side (dorsal opener). Cycles 6 and 7 show pronounced activity in the left ventral openers and activity in only the right ventral opener. Cycles 8, 9 and 10 show dominant muscle activity in the right dorsal opener with muscle activity reduced in the left dorsal and ventral opener muscles.

### The opener muscles

Recordings were made from all four opener muscles (left and right ventral and dorsal opener muscles) simultaneously. Two different types of rhythm were found to exist, and results show that the activity patterns between muscles of the

same group were not necessarily fixed. All four opener muscles were initially found to burst in phase and periods of opener muscle activity were followed by periods of inactivity, which represented closure of the ovipositor valves (Fig. 3.9A). During the same recording, periods of attenuated, or even absent opener muscle activity were observed in one side of the abdomen when opener muscle activity was present in another side of the abdomen (Fig. 3.9B). For example, attenuated opener muscle activity was observed in the right and left ventral opener and left dorsal opener muscles, while full opener muscle activity was observed in the right dorsal opener muscle (cycle number 5). This was followed by an absence of dorsal opener muscle activity but full opener muscle activity in the right and left ventral opener and left dorsal opener muscle (cycle number 6). This was then repeated for one more group of bursts (although attenuated opener muscle bursting returned for the right dorsal opener (cycle number 7). Isolated opener muscle activity then returned in the right dorsal opener (cycle number 8) (Fig. 3.9B). Out of a total of 15 recordings of all 4 opener muscles, 9 recordings showed similar examples of reciprocal opener muscle activation and inactivation.

**Closer and retractor muscles**

Recordings from the ventral closer and retractor muscles show that their activity occurs directly after opener muscle activity ceases (Thompson, 1986a; Seymour, 1990). This shows that the closing and retraction movements of the ovipositor valves occur simultaneously (Fig. 3.10A, B).

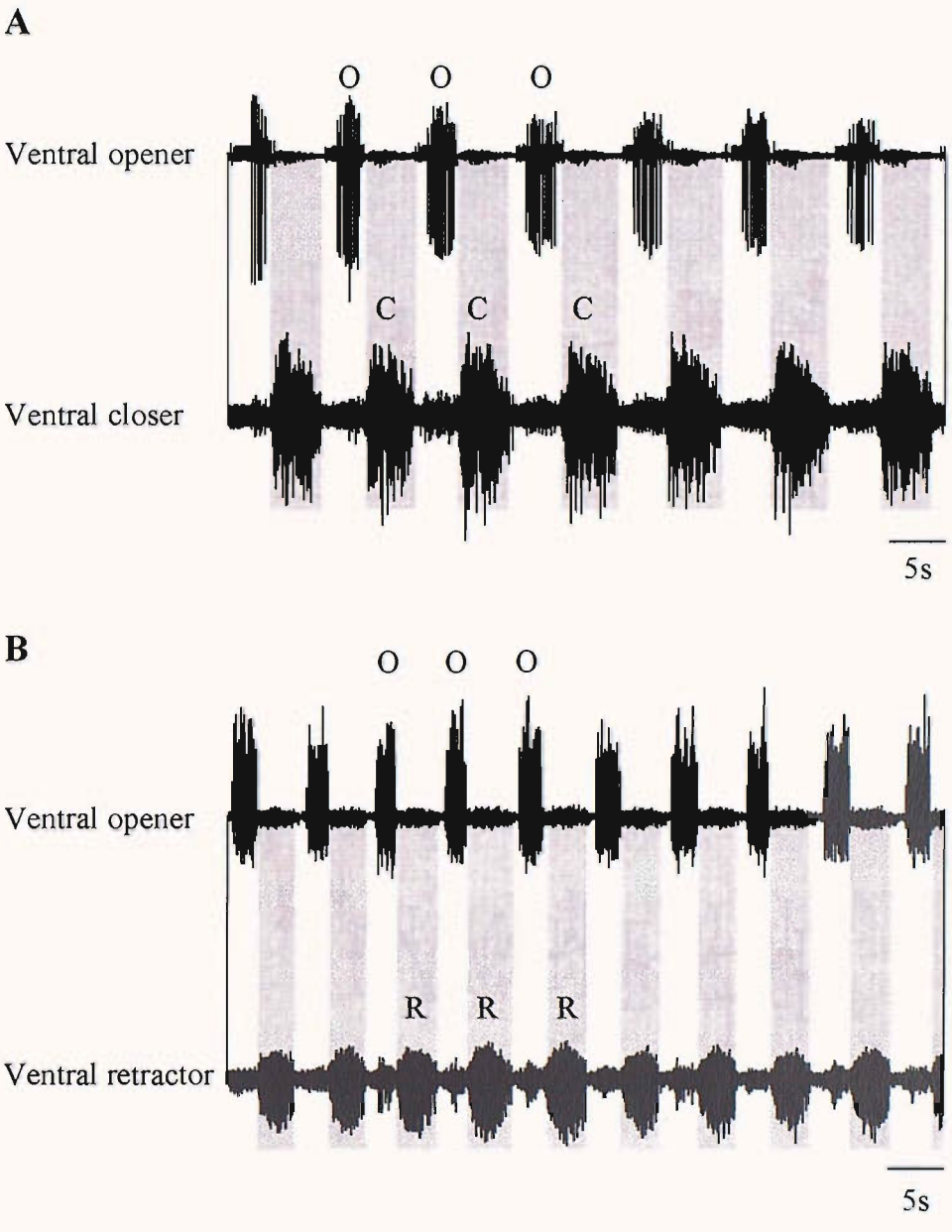



**Figure 3.10. The relationship between ventral opener, closer and retractor muscle activity. A.** A recording from a ventral opener and ventral closer muscle. The ventral closer muscle bursts (C) directly after the ventral opener muscle (O) in order to pull the valves shut. **B.** A recording from the ventral opener and ventral retractor muscle. The ventral retractor muscle bursts (R) directly after the ventral opener muscle. Note that the ventral closer and ventral retractor muscles are in phase and therefore active at the same time during a digging cycle. This shows that the valves of the ovipositor close and retract back into the abdomen in one combined movement.

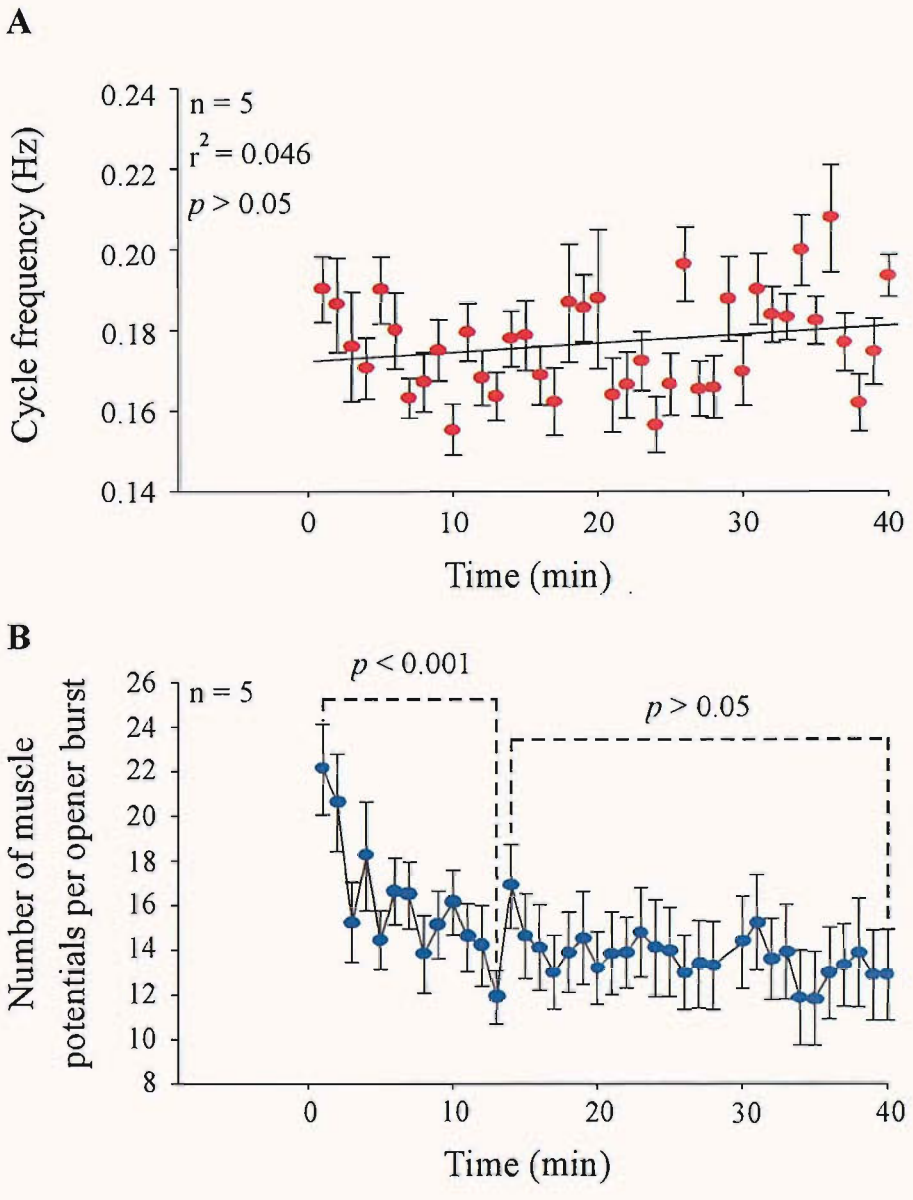



**Figure 3.11. The effect of time on opener muscle activity. A.** A 40 min recording shows no significant change in cycle frequency of the digging rhythm ($r^2 = 0.05$, $p > 0.05$). The digging rhythm oscillated about a value of approximately 0.17 ± 0.01 Hz (denoted by the regression line). **B.** The number of muscle potentials per opener burst declines significantly between the 1st and the 13th minute of the recording from 22.08 ± 2.03 muscle potentials per opener burst, to 11.88 ± 1.2 muscle potentials per opener muscle burst (Students T-test, $t = 4.3$, $p < 0.001$). No significant differences in the number of muscle potentials per opener muscle burst are observed between the 14th and 40th minute of the recording (Kruskal-Wallis, $K = 12.45$, $p > 0.05$).

### 3.3f Long-term recordings of the ventral opener muscles

Chapter 2 showed that the locust's choice of oviposition substrate is affected by its chemical composition and the concentration of the chemical within the substrate. This leads to the possibility that the isolated abdomen of the locust could

be a reliable preparation for further research into the behavioural responses to certain tastants. Moreover, the fact that locusts make behavioural choices between suitable and unsuitable oviposition sites suggests that the motor output of the digging rhythm needs to be modulated, either by the presence of neuromodulators within the central nervous system, or by sensory input, or by both. Long-term recordings of the ventral opener muscles were therefore performed to assess the stability of the preparation over time, by analysing the effect of time on cycle frequency, the number of muscle potentials per opener burst and duration of opener muscle activity. The opener muscles were exclusively used because of their large size and ease of recording. All experiments (n = 5 animals) were performed without any external sensory stimuli or exposure to known neuromodulators.

Over a time period of 40 min, the cycle frequency of the digging rhythm was found to oscillate about a value of approximately 0.18 Hz. The mean of 5 cycles were taken every minute for 40 min. The minimum cycle frequency of the digging rhythm was observed after 20 min of recording (0.16 ± 0.01 Hz, Mean ± S.E.M.) with the maximum cycle frequency being present after 36 min of recording (0.21 ± 0.01). The total mean cycle frequency (the mean of all the data points) was 0.18 ± 0.01 Hz. A linear regression analysis showed that there was no significant variation on the cycle frequency of the digging rhythm over time ($r^2 = 0.046$, $p > 0.05$) (Fig. 3.11A).

The number of muscle potentials in a ventral opener burst was also analysed over a recording duration of 40 min. There was an initial, statistically significant decrease in the number of muscle potentials of the ventral opener muscle during the first 13 min of recording. The number of muscle potentials per opener muscle burst decreased from a value of 22.08 ± 2.03 to a value of 11.88 ± 1.2 (Students T-test, t = 4.32, $p < 0.001$) (Mean ± S.E.M). Between 14 min and 40 min however, no

statistically significant variations in the numbers of muscle potentials per opener burst were observed (Kruskal-Wallis, $K = 12.45$, $p > 0.05$). Throughout a 40 min recording, there was a tendency for the number of muscle potentials of the ventral opener muscle to decrease. 3 out of 5 recordings from 5 different animals showed a decrease in the number of muscle potentials per opener muscle burst, over a period of 40 min (Fig. 3.11B).

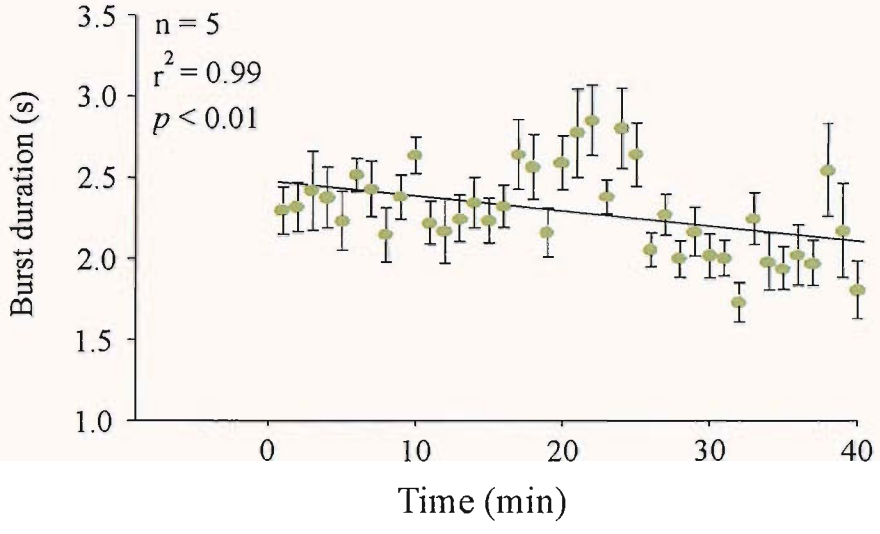



**Figure 3.12. The effect of time on the burst duration of the opener muscles.** The duration (s) of the opener muscle bursts significantly declined over a recording period of 40 min ($r^2 = 0.99$, $p < 0.01$).

The effect of recording duration on the burst duration of the opener muscle activity was also analysed. Although the ventral opener muscle burst duration remained relatively constant over the first 16 min of a recording, large fluctuations in the burst duration occurred, increasing between 19 and 25 min and then showing a large decrease after 25 min. A linear regression analysis shows a statistically significant decrease in burst duration over time ($r^2 = 0.99$, $p < 0.01$) (Fig. 3.12).

In summary, the number of muscle potentials in an opener muscle burst and burst duration changed over time however, cycle frequency remained constant.

## 3.4 Discussion

The purpose of this work was to assess the viability of isolated abdomen preparations for further research into the behavioural responses of the ovipositor valves to contact chemosensory input and in so doing, describe the basic parameters and phases of digging.

### 3.4a Variations in the patterns of activity of CPG's

Recordings from all four opener muscles showed variation in terms of their activation patterns. The dominant motor pattern was activity in all four opener muscles of the ovipositor valves that were normally in phase (as also observed by Thompson, 1986a). Alternating periods of opener muscle activity and inactivity were also observed, with activity of the opener muscles on one side and reduced or absent activity on the opposite side and vice versa. This showed that the CPG underlying oviposition digging is not fixed, but may be multifunctional. The behavioural relevance of these reciprocal periods of muscle activation and inactivation is unclear. Further behavioural and physiological analysis is therefore required in order to confirm a possible function for reciprocal periods of muscle activation and inactivation.

Although there is no evidence for the reciprocal activation and inactivation of the same muscle groups that produce the same movement in insects, there are examples in vertebrates. A possible reason for the lack of evidence in insects, could be that the majority of CPG research focuses on establishing the structure and network connections of CPG's. In order to establish detailed CPG structure, recordings from different muscle groups are necessary. Multiple recordings from different muscles of the same group do not provide this detail and may be rare for this reason. Recordings from hind limb flexor and hind limb extensor motor neurons

in the spinal cord of decerebrate cats have shown that despite muscle activity and movement of a leg, sub-groups of motor neurons innervating these muscle groups need not be active (La-freniere-Roula and McCrea, 2005). These periods of motor neuron inactivity are known as deletions and refer to a reduction or absence in the expected burst of a motor neuron or motor neuron pool. The reason for motor neuron inactivity but continued movement of the hind leg is unknown, but presumably results from the isolated inactivity of a sub unit of the CPG, while the rest of the CPG network remains active and can still produce a specific movement (Lafreniere-Roula and McCrea, 2005). The buccal CPG of the mollusc *Lymnaea* consists of 3 interneuronal sub-units (S1, S2 and S3), each of which are connected to a set of motor neurons that produce the rhythmic, tri-phasic (protract, rasp, retract) feeding movements of the mouthparts (Rose and Benjamin, 1979) but can also show variability. For example, S1 and S2 sub units of the CPG network can be suppressed with isolated neural activity in sub-unit S3, which produces regurgitation movements of the mouthparts (Quinlan and Murphy, 1996). Clearly variability in rhythmic motor patterns is common across different species, and the patterns of activity found in the locust oviposition CPG reflect this common observation.

### 3.4b The effect of time on the cycle frequency, number of muscle potentials per opener burst and opener muscle burst duration

The cycle frequency of centrally generated rhythmic behaviours has previously been shown to vary with time. In the respiratory system of the crab, recordings from nerves that innervate the gill bailer and the scaphognathite, have shown that the cycle frequency of the CPG has a range of 0.47 to 3.3 Hz (DiCaprio *et al.* 1997). Similarly, when recording from mandibular nerves that produce the rhythmic feeding movements of *Manduca sexta*, the cycle period varied from

between 3 and 45 seconds (cycle frequency; 0.02 to 0.3 Hz) (Bowdan and Wye, 2000). Although there seems to be no obvious reason for the large fluctuations in the cycle frequency of the oviposition digging rhythm over time, one reason could be a lack of mechanical contact with a substrate when using isolated abdomen preparations. In intact animals, the ovipositor valves are in frequent contact with a substrate while digging. When intact animals are rhythmically digging an egg-laying substrate, continued rhythmic deflections of mechanosensory hairs located on the exterior surface of the valves provide constant rhythmic sensory input to the CPG that underlies the digging behaviour. Without physical contact with an egg-laying substrate, rhythmic deflections of the mechanosensory hairs are absent. Thompson (1986b) showed that decapitation of locusts resulted in periods of erratic bursting from the opener muscles, although periods of erratic muscle activity were followed by regular, rhythmic opener muscle activity (Thompson, 1986b). This suggests that the lack of descending control over the rhythmic movements of the ovipositor valves as a result of decapitation, results in a digging rhythm that is not the same as in an intact animal and explains the fluctuations in cycle frequency. Despite this finding, subsequent long-term recordings of the opener muscles by Seymour (1990) showed little variation in cycle frequency. Over the course of a 2 hr experiment, the cycle frequency was recorded at 30 min intervals and no significant increases or decreases in the cycle period were observed (Seymour, 1990). Despite the oscillation of cycle frequency over the course of 40 min as observed in this study, and over the course of 2 hr as observed by Seymour (1990) the cycle frequency in both studies did not significantly increase or decrease (Seymour, 1990) and therefore represents an ideal preparation with which to investigate the effects of chemical stimulation on the oviposition rhythm.

The number of muscle potentials per opener muscle burst and the burst duration of the opener muscles did show variation over a recording period of forty minutes. This suggests that as the duration of experiments progressed, the quality of the preparation may have gradually degenerated as a result of the abdomen not being part of a freely behaving animal. The reduction in number of muscle potentials suggests that the number of motor neurons or their spike number that were activating the opener muscles, may have gradually decreased as the preparation degenerated. A similar decrease was also observed for opener muscle burst duration, further reinforcing the possibility that the motor output of the opener muscles gradually changes over time. Recordings from the left ventral opener muscle over a time period of 45 min by Belanger and Orchard (1993) have shown a similar significant decrease in opener muscle burst duration. In contrast however, recordings from intact animals show that no significant decrease in opener muscle burst duration occur over a similar time period, suggesting that isolating the abdomen results in a degeneration of the preparation as the duration of a recording increases possibly as a result of anoxia. For example, muscle recordings from the pyloric muscles of the lobster, *Hommarus gammarus*, have shown that the cycle period of muscle activity can increase from a period of 2-3s to a period length of 10s under artificially imposed anoxic conditions (Clemens *et al.* 1999).

Another possibility is that the oviposition digging rhythm showed a degree of adaptation over the 40 min time period. Although rhythmic exteroceptive sensory input is not necessary for the production of rhythmic movements, proprioceptive sensory inputs produced as a result of the movement of the ovipositor valves may be necessary for their maintenance and this may decrease over time. Further experiments are needed to resolve this issue.

### 3.4c Rhythmic movement and chemosensory inputs

Locusts need to show an ability to discriminate between suitable and unsuitable oviposition sites. Chapter 2 has shown that chemical composition of an oviposition site can markedly affect substrate choice. Kalogianni (1995) showed that certain hairs on the ovipositor of locusts responded to mechanical stimulation. Moreover, Woodrow (1965) has shown an effect of certain salts on the oviposition behaviour of migratory locusts with 2 M NaCl stopping the rhythmic movements of the ovipositor valves.

The effect of chemosensory input on CPG's has not been well studied. To date, invertebrate preparations have predominantly been used. The rhythmic feeding movements of *L. stagnalis* can be initiated via stimulation of the mouthparts with 100mM sucrose (Whelan and McCrohan, 1996). Stimulation of the mouthparts with sucrose solution results in a depolarisation of protractor motor neurons (Elphick *et al.* 1995) that initiates a protractive movement of the mouthparts. As the rhythmic depolarisation of the protractor motor neurons continues, the rasp and retractor motor neurons are gradually recruited until the full feeding rhythm is observed (Benjamin and Elliott, 1985).

The above research raises the possibility that the digging rhythm of locusts may respond to chemosensory input. Behavioural assays have shown that a locust's choice of an oviposition site is affected by the chemical nature of the substrate. Tousson and Hustert (2000) confirmed that basiconic sensilla are present upon the ovipositor valves of locusts. Despite these observations however, there have been no investigations into the effects of various chemicals on the rhythmic digging movements of the ovipositor valves.

# Chapter 4 – The effect of chemical stimulation on the oviposition digging rhythm

Chapter 4

# The effect of chemical stimulation on the oviposition digging rhythm

## 4.1 Introduction

All animals have the ability to modify their behaviour in response to a varying external environment (Kien, 1977). Different sensory receptors located on and in the body surface perform the function of encoding information about the animals' environment. This information is subsequently processed within the central nervous system and an appropriate behavioural response produced (Ware *et al.* 1975). For example, detailed studies of how behavioural responses are modified in response to different sensory inputs include the escape responses of crustaceans and insects (Wine *et al.* 1975; Cromarty *et al.* 1991; Casagrand and Ritzmann, 1992; Ye *et al.* 2003), the whole-body withdrawal movements of molluscs (Arshavsky *et al.* 1994; Zhukov and Kononenko, 2002) the modification of insect flight and locomotion (Kutsch *et al.* 1994; Wolf, 1992) and behavioural responses to contact chemoreception and olfactory stimuli in vertebrates and invertebrates (Spector, 2000; Buck, 1996; Rogers and Newland, 2000; Hildebrand, 1995).

### 4.1a Chemosensory input

In invertebrates, contact chemosensory input plays a crucial role in modifying behaviour (Roessingh *et al.* 1997). In molluscs, chemical stimulation of the mouthparts with sucrose solutions has been shown to initiate rhythmic feeding movements of the mouthparts (Kemenes *et al.* 1986). In locusts, contact chemosensory stimulation of the hind legs with a variety of different chemicals at different concentrations has been shown to result in a leg withdrawal response

(Newland, 1998; Rogers and Newland, 2000). Chemical cues can therefore be detected by contact chemoreceptors, the basiconic sensilla, that are located on the tarsi and hind legs of the locust (White and Chapman, 1990; Newland and Burrows, 1994). Information about a chemical stimulus is transduced at the dendrites of the sensory neurons located within the shafts of a sensillum, and nerve impulses are propagated to the central nervous system where the signal is processed within the metathoracic ganglion, and where neural networks controlling leg movements are located (Newland, 1999; Rogers and Newland, 2002). When the hind legs of locusts are in contact with concentrations of chemicals that are aversive, motor neurons that are part of the neural networks controlling leg movement, function to withdraw the hind leg away from the chemical stimulus. If the stimulus is non-aversive, the hind leg remains in the same position. Rogers and Newland (2000) showed that chemicals that represented dietary components and anti-feedants had the ability to act aversively provided the chemicals were applied at a sufficiently high concentration. For example, applying droplets of dietary components such as the carbohydrate sucrose, and the amino acid salt LG to the hind legs, both resulted in the locust withdrawing its leg away from the stimulus. Similar results were observed for the anti-feedant NHT and the salt NaCl. As the concentration of all chemicals was increased, the probability that a locust would withdraw its leg increased (Rogers and Newland, 2000).

A similar concentration dependent effect was shown in the choice of oviposition site (see *Chapter 2*; Woodrow, 1965). Tousson and Hustert (2000) showed that basiconic sensilla were also present on the ovipositor valves of locusts and responded to chemical stimulation. Experiments by Kalogianni (1995, 1996) have also shown that the ovipositor is covered by a number of different hairs that respond to touch. Moreover, Kalogianni and Burrows (1996) showed that

mechanosensory information is integrated and processed within an area of the locust central nervous system, termed the terminal abdominal ganglion, that produces the rhythmic digging movements of the ovipositor valves (Kalogianni and Burrows, 1996). While spiking local interneurons in the metathoracic ganglion of the locust have been shown to process both mechanosensory and chemosensory signals upon mechanosensory and chemosensory stimulation of the hind legs (Newland, 1999), we know little of the effect of contact chemosensory stimulation of the ovipositor valves on the neural circuitry generating behavioural responses of the valves.

The previous chapter showed that severing the abdomen from the rest of the locusts' body could reliably elicit rhythmic digging movements of the ovipositor valves. Chapter 2 showed that oviposition was dependent on the chemical composition of the egg-laying substrate. The aim of this chapter is to ask if chemosensory stimulation influences the oviposition digging rhythm. Two key parameters, the cessation of the oviposition digging rhythm and the cycle frequency on return of the rhythm, were analysed in order to determine whether concentration dependent effects of chemicals were present.

## 4.2 Materials and Methods

### 4.2a Locusts

Female desert locusts, *Schistocerca gregaria* (Forskål) maintained and reared as described in *Chapter 2*.

### 4.2b Chemical stimulation of the ovipositor valves

An oviposition digging rhythm is usually absent in intact locusts and is thought to be controlled by descending inhibition (Thompson, 1986b). Initiation of the digging rhythm is therefore caused by a release from descending inhibition which can be carried out by decapitating locusts with a twisting motion and a sharp pull that serves to remove the gut. This procedure reliably produced rhythmic movements of the ovipositor valves similar to those seen in intact female locusts that have been defined as 'fictive digging movements' by Thompson (1986a).

The abdomen was then isolated from the thorax and pinned laterally (see Fig. 3.1, *Chapter 3*) to a Plasticine stage with the ovipositor valves overhanging the edge of the stage. This allowed chemicals to drain away from the valves and served to prevent constant chemosensory input. The anterior end of the abdomen was constantly perfused with fresh locust saline throughout an experiment. Individual droplets of chemical were applied to the ventral surface of the ovipositor valves using a Pasteur Pipette (Fig. 4.1). This area of the ovipositor valves was selected because the number and density of basiconic sensilla is highest (Tousson and Hustert, 2000).

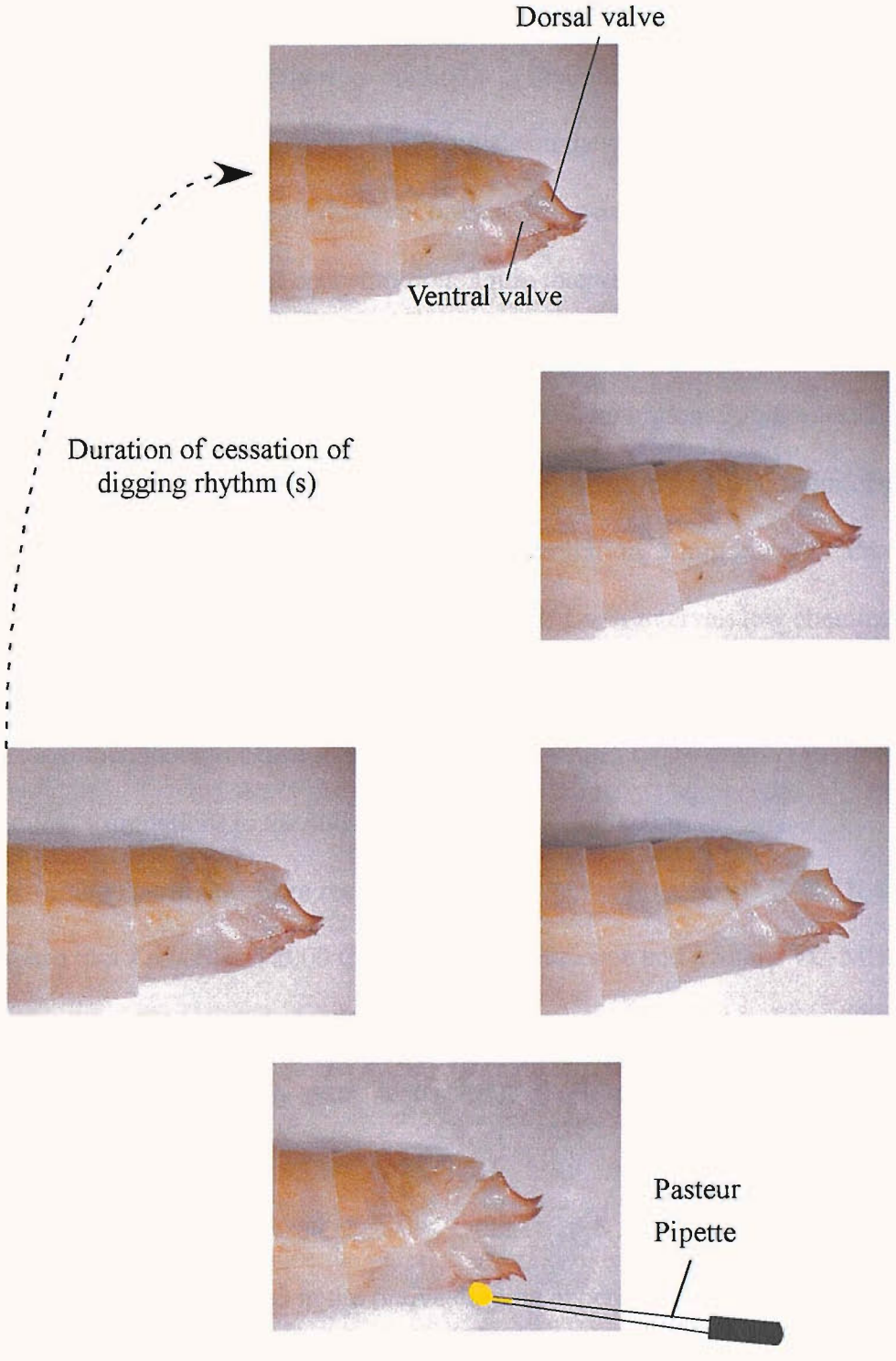



**Figure 4.1. Chemical stimulation of the ovipositor valves.** Droplets of chemicals were applied to the ventral surface of the ovipositor valves using a Pasteur Pipette. To minimize the effect of mechanical stimulation on the digging rhythm, droplets were always applied to the ventral surface of the ovipositor valves during the opening phase of a digging cycle. The effect of chemical stimulation on the duration of the cessation of the rhythm and the cycle frequency once the digging rhythm returned was then analysed.

Experiments with each chemical involved 2 different concentrations and the order in which the chemicals were applied was randomized to remove any potential effect of order of presentation of the chemicals on the digging rhythm. Chemicals were therefore presented in 4 different orders: Protocol 1; distilled water (DW), 1 min interval, low chemical concentration, 3 min interval, DW, 1 min interval, high chemical concentration, 3 min interval. Protocol 2; low chemical concentration, 3 min interval, DW, 1 min interval, high chemical concentration, 3 min interval, DW, 1 min interval. Protocol 3; DW, 1 min interval, high chemical concentration, 3 min interval, DW, 1 min interval, low chemical concentration, 3 min interval. Protocol 4; high chemical concentration, 3 min interval, DW, 1 min interval, low chemical concentration, 3 min interval, DW, 1 min interval. The durations of the intervals, represent the amount of time in minutes from the return of the digging rhythm (after chemical stimulation) to the subsequent chemical application.

All experiments were recorded with a colour video camera (JVC TK-C1380) attached to a Zeiss Stemi 2000 CS binocular microscope and a Sharp VC-MH704 video recorder. Results were analysed with the aid of a For-A video timer that provided a timing accuracy to the nearest $100^{th}$ of a second.

### 4.2c Test chemicals

The chemicals used in the fictive digging experiments were chosen based on their relevance to locust behaviour and for their representation of the basic taste qualities. NaCl represents an anionic salt taste quality. Sucrose and LG represent the macronutrients carbohydrate and amino acids, respectively, and are key components of the locusts' diet. Both chemicals are known to promote feeding (Simpson and Raubenheimer, 1993a). HQ and NHT are bitter tasting, toxic anti-feedants that discourage feeding related behaviours in insects (White and Chapman,

1990; Chapman *et al.* 1991). TA is a secondary plant compound that represents a sour tasting chemical stimulus.

The concentrations used varied for each chemical and the effective ranges were estimated by performing preliminary experiments. All chemicals were made up to their required concentration in distilled water. The concentrations of the test chemicals used were as follows: NaCl (Fisher Chemical Co), 10mM and 250mM. Sucrose (Fisher Chemical Co), 100mM and 1 M. LG (Sigma Aldrich Chemical Co), 50mM and 2 M. HQ (Sigma Aldrich Chemical Co), 10mM and 100mM. NHT (Sigma Aldrich Chemical Co), 0.5mM and 2.5mM) and TA (Sigma Aldrich Chemical Co), 5mM and 75mM.

### 4.2d Physiological methods

Recordings of the muscles were obtained as described in *Chapter 3*.

### 4.2e Statistical methods

Differences between the duration of rhythm cessation for control, low and high chemical concentrations were tested using Students T-tests. A Students T-test was also used to test the effects of chemical concentration on the subsequent cycle frequency on return of the rhythm.

## 4.3 Results

Droplets of 6 chemicals each at 2 different concentrations were applied to the ventral surface of the ovipositor valves of isolated abdomen. The effect of chemical type and concentration on 2 parameters of the digging rhythm; the duration of rhythmic cessation and the cycle frequency on return of the rhythm was then analysed.

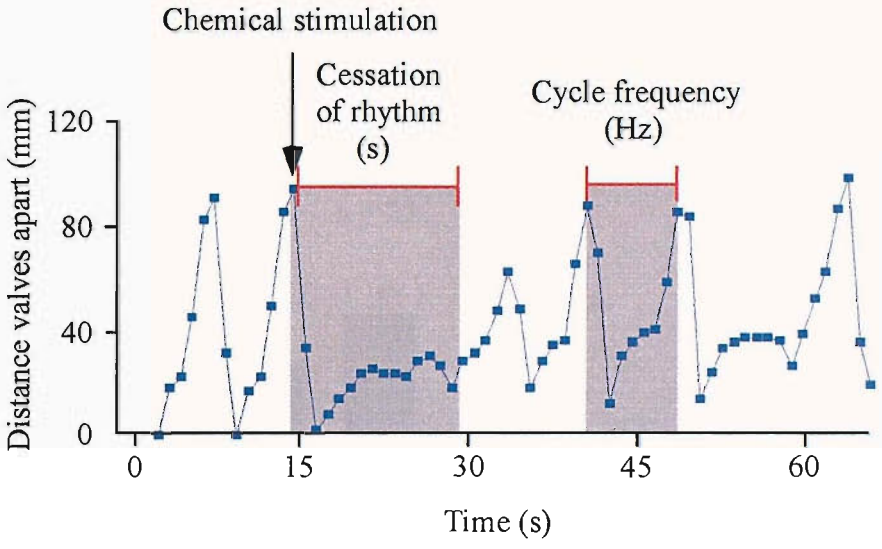



**Figure 4.2. The effect of chemical stimulation of the ovipositor valves on the cessation of the digging rhythm.** All chemical stimulations of the ovipositor valves resulted in a cessation of the digging rhythm. The effect of chemical type and concentration of different chemicals on the duration of cessation of the rhythm, and subsequent cycle frequency on its return were analysed.

### 4.3a The effect of chemical stimulation on the duration of cessation of the digging rhythm

***Dietary requirements.*** All chemical applications to the ovipositor valves, including distilled water controls, resulted in a cessation of the rhythm (Fig 4.2). No statistically significant differences were observed between distilled water applications and low chemical concentrations for all dietary requirements tested (control; 25.34 ± 1.69s, 100mM sucrose; 29.29 ± 2.77s, Students T-test, t = -1.28, *p*

> 0.05, control; 19.52 ± 1.73s; 50mM LG; 16.02 ± 2.18s, t = 0.55, $p > 0.05$, control; 30.69 ± 1.75s, 10mM NaCl; 30.22 ± 2.47s, t = 0.15, $p > 0.05$).

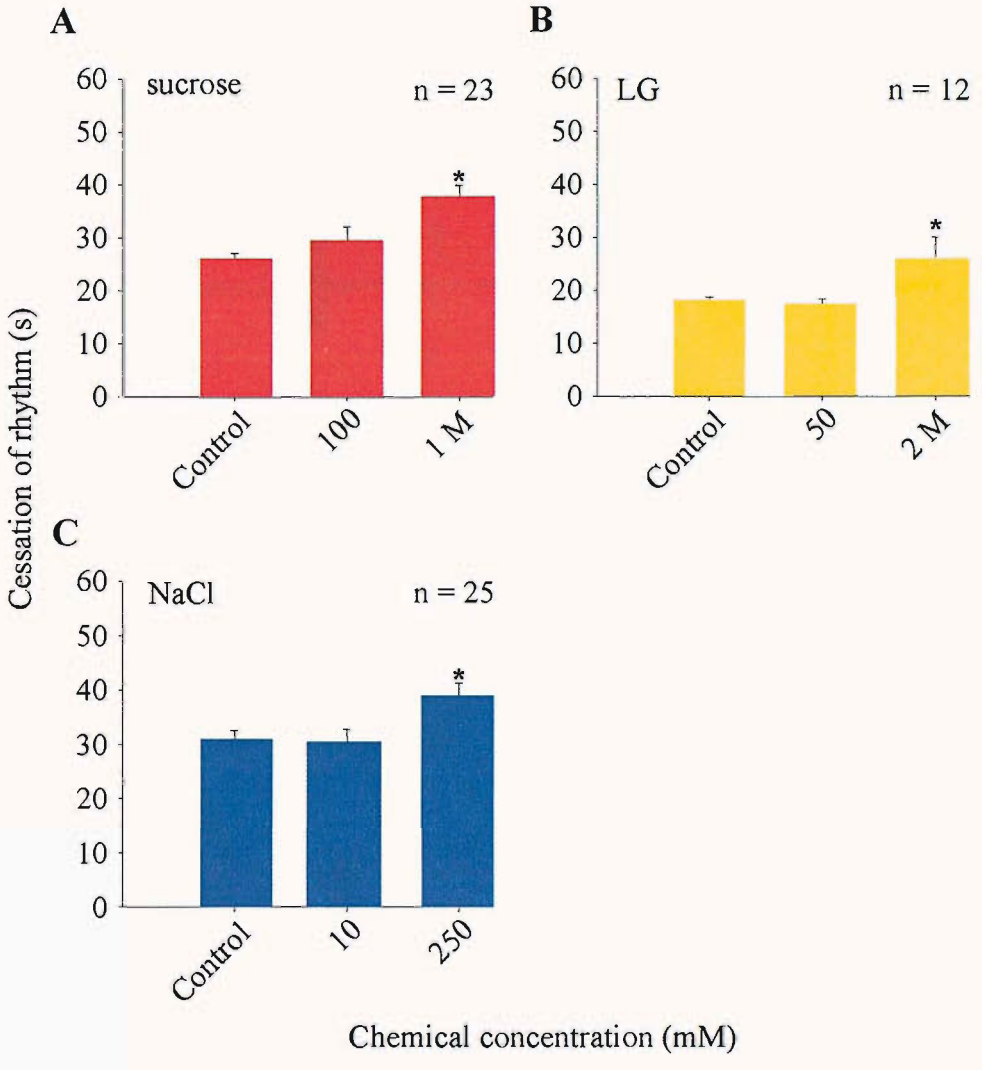



**Figure 4.3. The effect of chemical stimulation on duration of cessation of the digging rhythm. A.** 1 M sucrose increased the cessation of the rhythm for significantly longer than 100mM sucrose and a distilled water control (control; 24.34 ± 1.69s, 100mM sucrose; 29.29 ± 2.77s, 1 M sucrose; 37.18s ± 2.67s; Students T-test, t = -2.05, $p < 0.05$). **B.** Similarly, 2 M LG caused a longer cessation of the rhythm than 50mM LG and controls (control; 19.52 ± 1.73s, 50mM LG; 16.02 ± 2.18s, 2 M LG; 25.8 ± 4.14s; Students T-test, t = -2.07, $p < 0.05$). **C.** Stimulating the ovipositor valves with 250mM NaCl caused a longer cessation than 10mM NaCl and distilled water controls (control; 30.69 ± 1.75s, 10mM NaCl; 30.22 ± 2.47s, 250mM NaCl; 38.35 ± 2.86s; Students T-test, t = 2.14, $p < 0.05$) (* $p < 0.05$).

High concentrations of all dietary requirements applied to the ovipositor valves resulted in statistically significant increases in the duration of cessation of the rhythm, compared to their respective low chemical concentrations and distilled water controls (1 M sucrose; 37.18 ± 2.67s, 100mM sucrose; 29.29 ± 2.77s, t = -2.05, $p <$

0.05, control; 25.34 ± 1.69s, t = -3.89, $p < 0.01$, 2 M LG; 25.8 ± 4.14s, 50mM LG; 16.02 ± 2.18s, t = -2.07, $p < 0.05$, control; 19.52 ± 1.73s, t = -2.45, $p < 0.05$, 250mM NaCl; 38.35 ± 2.86s, 10mM NaCl; 30.22 ± 2.47s, t = -2.14, $p < 0.05$, control; 30.69 ± 1.75s, t = -2.4, $p < 0.05$ (Fig. 4.3A, B, C; Fig 4.4A, B).

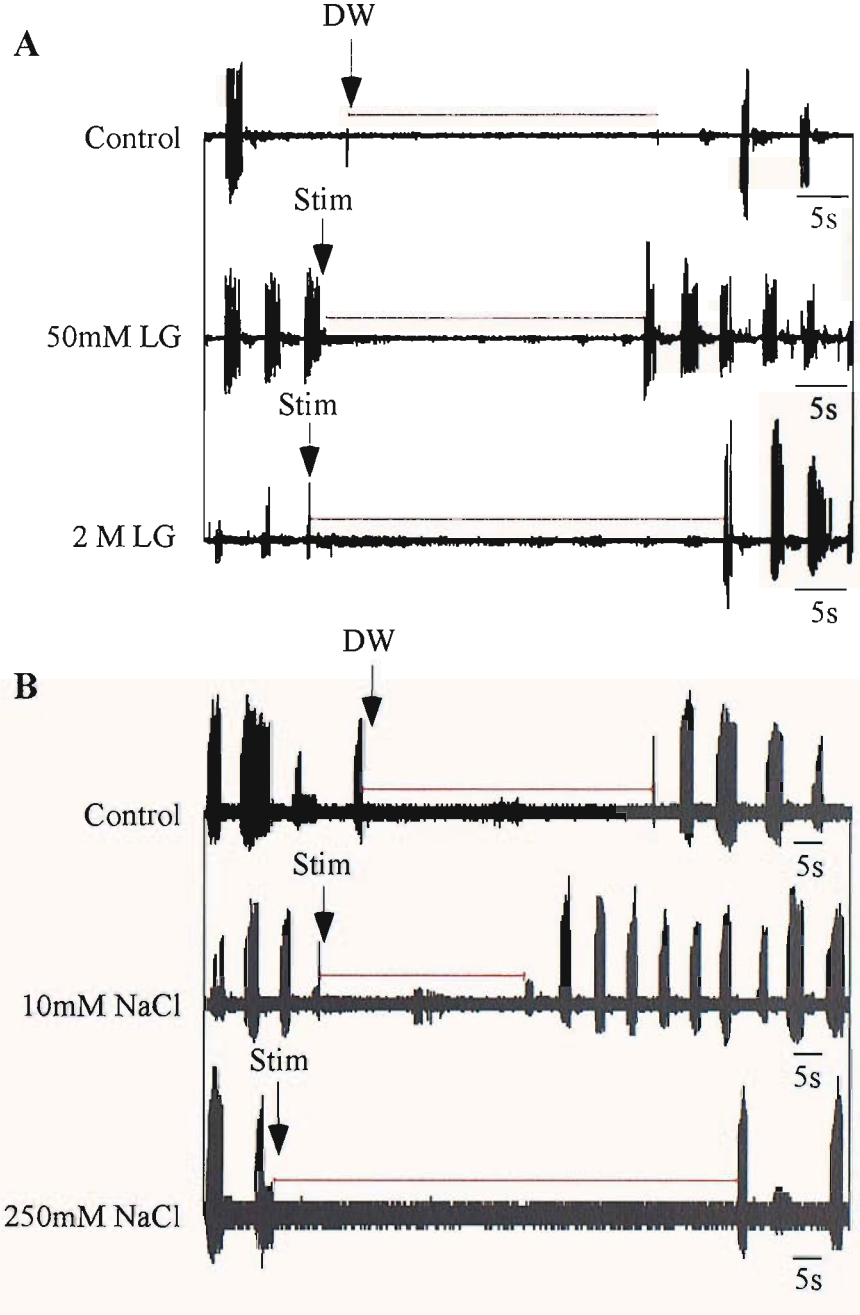



**Figure 4.4. The effect of chemical stimulation on the activity of the opener muscles. A.** An example showing that chemically stimulating the ovipositor valves with 2 M LG resulted in a cessation of opener muscle activity for a longer duration compared to distilled water and 50mM LG applications. **B.** Similarly, 250mM NaCl resulted in opener muscle activity cessation for longer than distilled water and 10mM NaCl. Although all chemical applications to the ovipositor valves stopped the digging rhythm, high concentrations of a given chemical stopped the rhythm for longer.

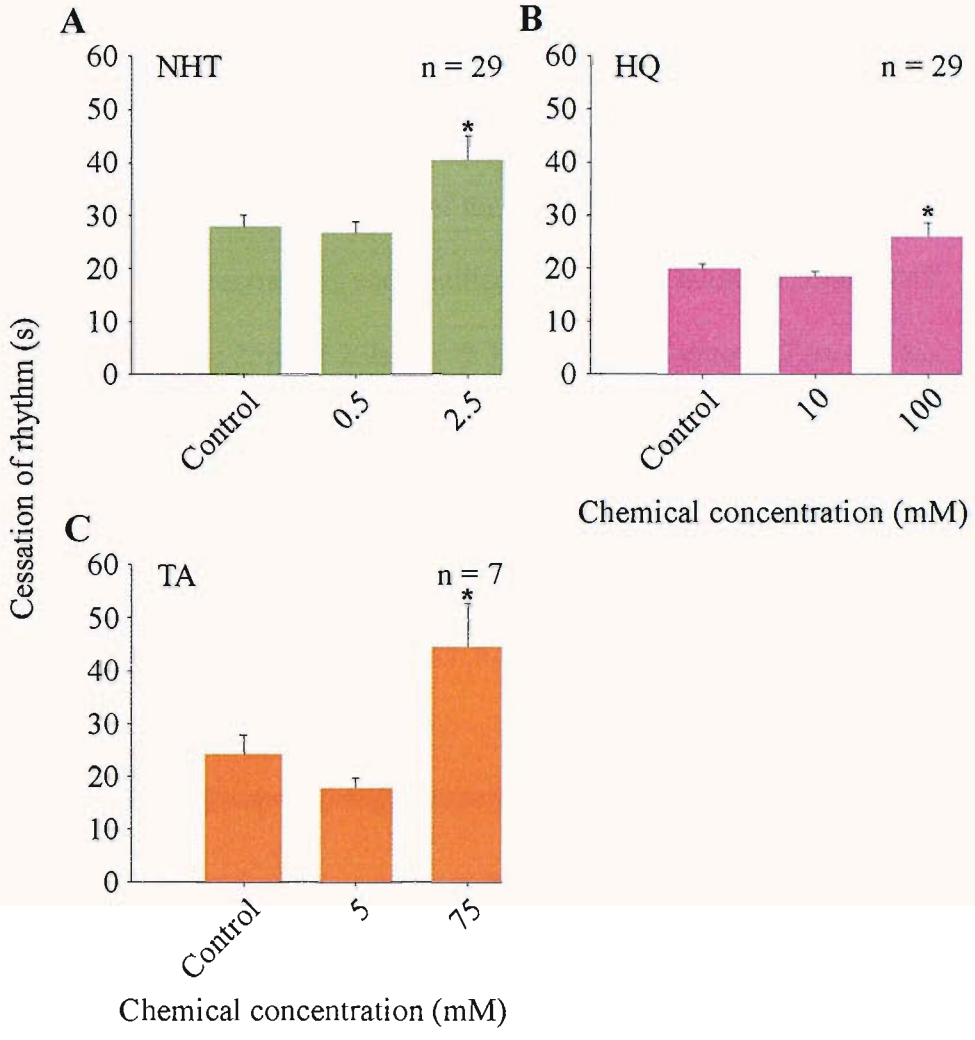



**Figure 4.5. The effect of chemical stimulation on the duration of cessation of the digging rhythm. A.** 2.5mM NHT caused a longer cessation compared to 0.5mM NHT and distilled water controls (control; 27.64 ± 2.46s, 0.5mM NHT; 25.8 ± 2.9s, 2.5mM NHT; 39.67 ± 5.14s; Students T-test, t = -2.39, $p < 0.05$). **B.** 100mM HQ caused a longer cessation of the rhythm than 10mM HQ and distilled water controls (control; 19.82 ± 0.88s, 10mM HQ; 17.96 ± 1.25s, 100mM HQ; 27.36 ± 3s; Students T-test, t = -2.89, $p < 0.01$). **C.** 75mM TA caused longer cessation than 5mM TA and controls (control; 15.9 ± 3.69s, 5mM TA; 23.55 ± 4.23s, 75mM TA; 43.86 ± 8.78s; Students T-test, t = -2.8, $p < 0.01$) (* $p < 0.05$).

***Anti-feedants.*** All HQ, NHT and TA applications to the ovipositor valves also resulted in cessation of the rhythm. No statistically significant differences in duration of cessation of the rhythm were observed between distilled water applications and low concentrations of all anti-feedants (control; 19.82 ± 0.88s, 10mM HQ; 17.96 ± 1.25s, t = 0.69, $p > 0.05$, control; 27.64 ± 2.46s, 0.5mM NHT;

25.81 ± 2.9s, t = 0.45, $p > 0.05$, control; 23.55 ± 4.23s, 5mM TA; 15.9 ± 3.69s, t = 1.13, $p > 0.05$).

Conversely, high concentrations of all anti-feedants resulted in statistically significant increases in the duration of the cessation of the rhythm compared to their low respective concentrations and distilled water applications (2.5mM NHT; 39.67 ± 5.14s, 0.5mM NHT; 25.81 ± 2.9s, t = -2.35, $p < 0.05$, control; 27.64 ± 2.46s, t = -2.39, $p < 0.05$, 100mM HQ; 27.36 ± 3s, 10mM HQ; 17.96 ± 1.25s, t = -2.89, $p < 0.05$, control; 19.82 ± 0.88s, t = -3.06, $p < 0.05$, control; 27.64 ± 2.46s, t = -2.39, $p < 0.05$, 75mM TA; 43.86 ± 8.78s, 5mM TA; 15.9 ± 3.69s, t = -2.8, $p < 0.05$, control; 23.55 ± 4.23s, t = -2.36, $p < 0.05$) (Fig. 4.5A, B, C; Fig 4.6, 4.7).

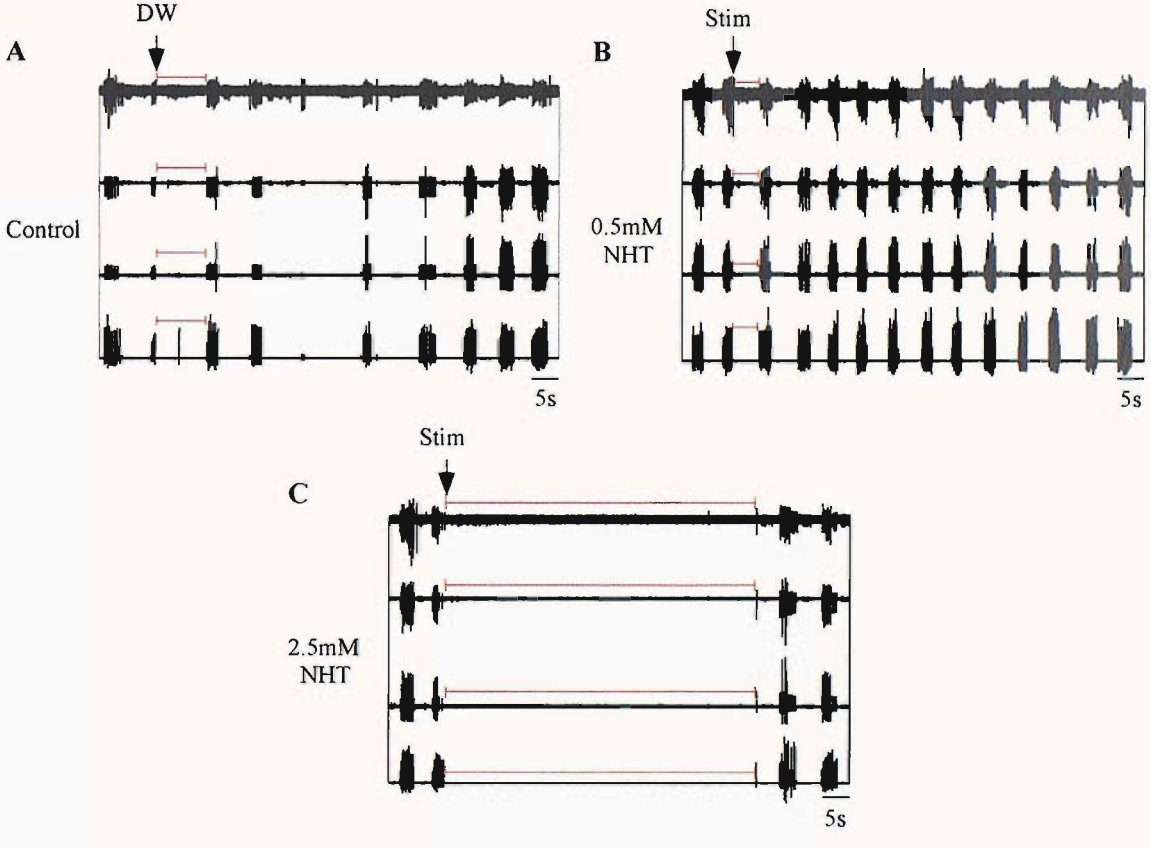



**Figure 4.6. The effect of the anti-feedant NHT on opener muscle activity. A.** An example showing a recording from all 4 opener muscles shows that distilled water resulted in the cessation of all 4 opener muscles. **B.** A low (0.5mM) concentration of NHT showed no significant effect of chemical stimulation on the duration of cessation of the rhythm. **C.** A 2.5mM concentration of NHT resulted in an increase in the duration of the cessation of the rhythm compared to 0.5mM NHT concentrations of NHT and distilled water controls.

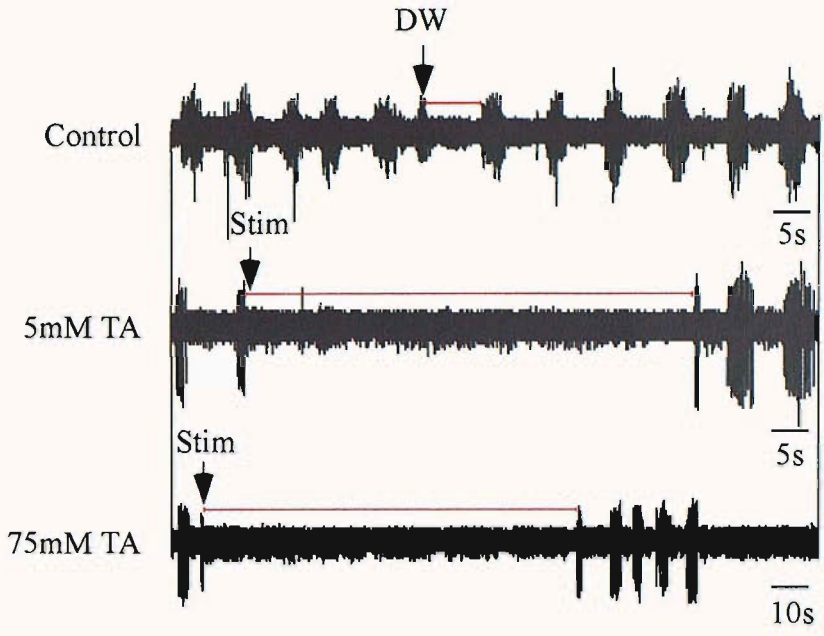



**Figure 4.7. The effect of the anti-feedant TA on opener muscle activity.** An example showing that a 75mM concentration of TA caused a cessation of opener muscle activity for longer durations compared to distilled water and 5mM TA applications to the ovipositor valves. Note that the time scale of the 75mM TA trace is 10s compared to the 5s time scales of the distilled water and 5mM TA trace.

Concentration dependent effects on the duration of the cessation of the rhythm were therefore observed for all chemicals tested. Moreover, the concentration at which a chemical became aversive depended on the type of chemical. Known anti-feedants (NHT, TA and HQ) acted to stop the rhythm at lower concentrations compared to chemicals that represented dietary requirements (NaCl, sucrose and LG) (Fig. 4.3, 4.4).

### 4.3b The effect of chemical stimulation on the cycle frequency of the digging rhythm

***Dietary requirements.*** The effect of chemical stimulation on the cycle frequency of the digging rhythm on its return following chemical stimulation was also analysed. For all dietary requirements tested, no effect of chemical type or concentration on the

cycle frequency of the digging rhythm was observed (Fig. 4.8A, B, C). (control; 0.18 ± 0.003 Hz, 100mM sucrose; 0.18 ± 0.005 Hz, Students T-test, t = 0.59, $p >$ 0.05, 1 M sucrose; 0.18 ± 0.004 Hz, t = 0.56, $p > 0.05$), LG (control; 0.14 ± 0.004 Hz, 50mM LG; 0.15 ± 0.006 Hz, t = -0.75, $p > 0.05$, 2 M LG; 0.15 ± 0.005 Hz, t = -0.91, $p > 0.05$) and NaCl (control; 0.14 ± 0.002 Hz, 10mM NaCl; 0.15 ± 0.003 Hz, t = -0.81, $p > 0.05$, 250mM NaCl; 0.16 ± 0.005 Hz, t = -1.27, $p > 0.05$).

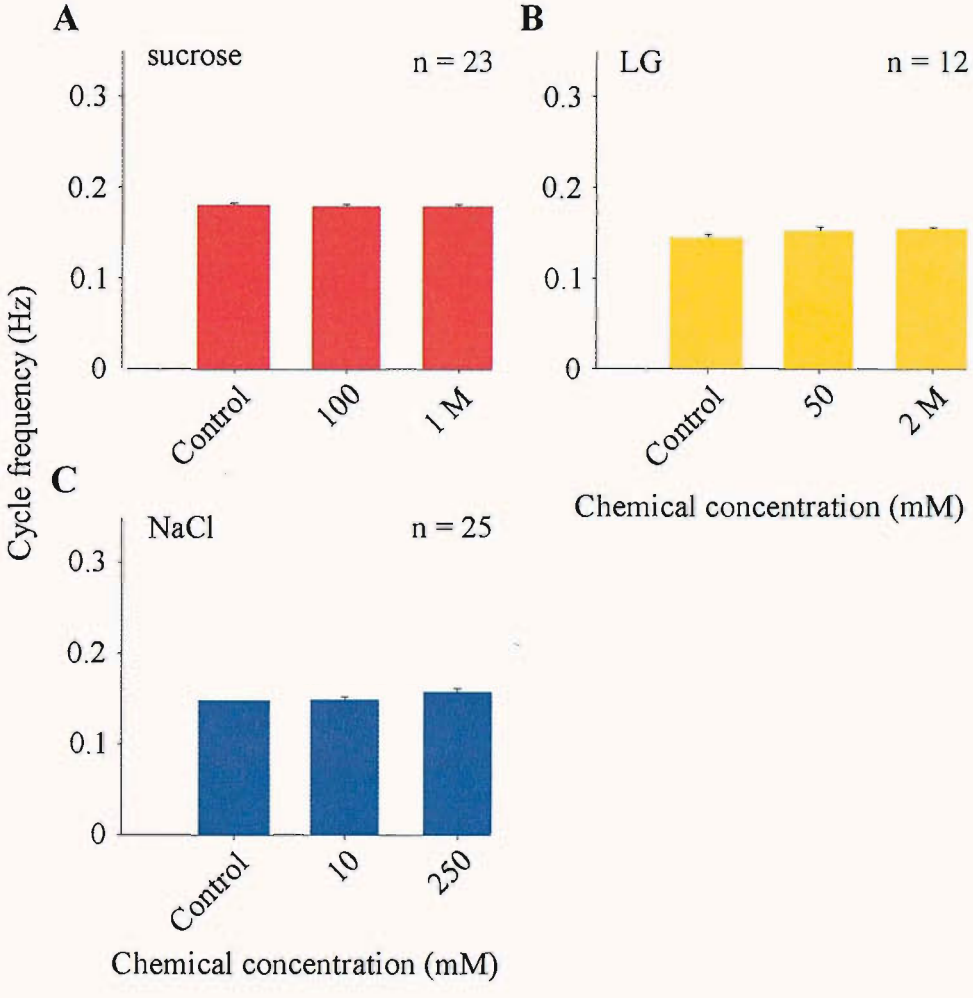



**Figure 4.8. The effect of chemical stimulation on the cycle frequency of the digging rhythm. A.** Chemical stimulation of the ovipositor valves with distilled water, 100mM and 1 M sucrose showed no statistically significant effect on the rhythm. **B.** No statistically significant effect of LG on the cycle frequency was observed between control, 50mM and 2 M LG applied to the ovipositor valves. **C.** Similarly, distilled water, 10mM and 250mM NaCl applied to the ovipositor valves had no concentration dependent effect on the cycle frequency of the digging rhythm ($p > 0.05$).

***Anti-feedants.*** Similarly, for all anti-feedants tested and at a range of concentrations, no effect on cycle frequency following cessation of the rhythm was observed; HQ (control; 0.15 ± 0.002 Hz, 10mM HQ; 0.24 ± 0.085 Hz, t = -1.48, $p > 0.05$, 100mM HQ; 0.15 ± 0.003 Hz, t = -1.44, $p > 0.05$), NHT (control; 0.15 ± 0.003, 0.5mM NHT; 0.15 ± 0.005 Hz, t = -0.54, $p > 0.05$, 2.5mM NHT; 0.14 ± 0.004 Hz, t = 1.22, $p > 0.05$) and TA (control; 0.16 ± 0.006 Hz, 5mM TA; 0.17 ± 0.007 Hz, t = 0.84, $p > 0.05$, 75mM TA; 0.15 ± 0.02 Hz, t = 0.79, $p > 0.05$) (Fig. 4.9A, B, C).

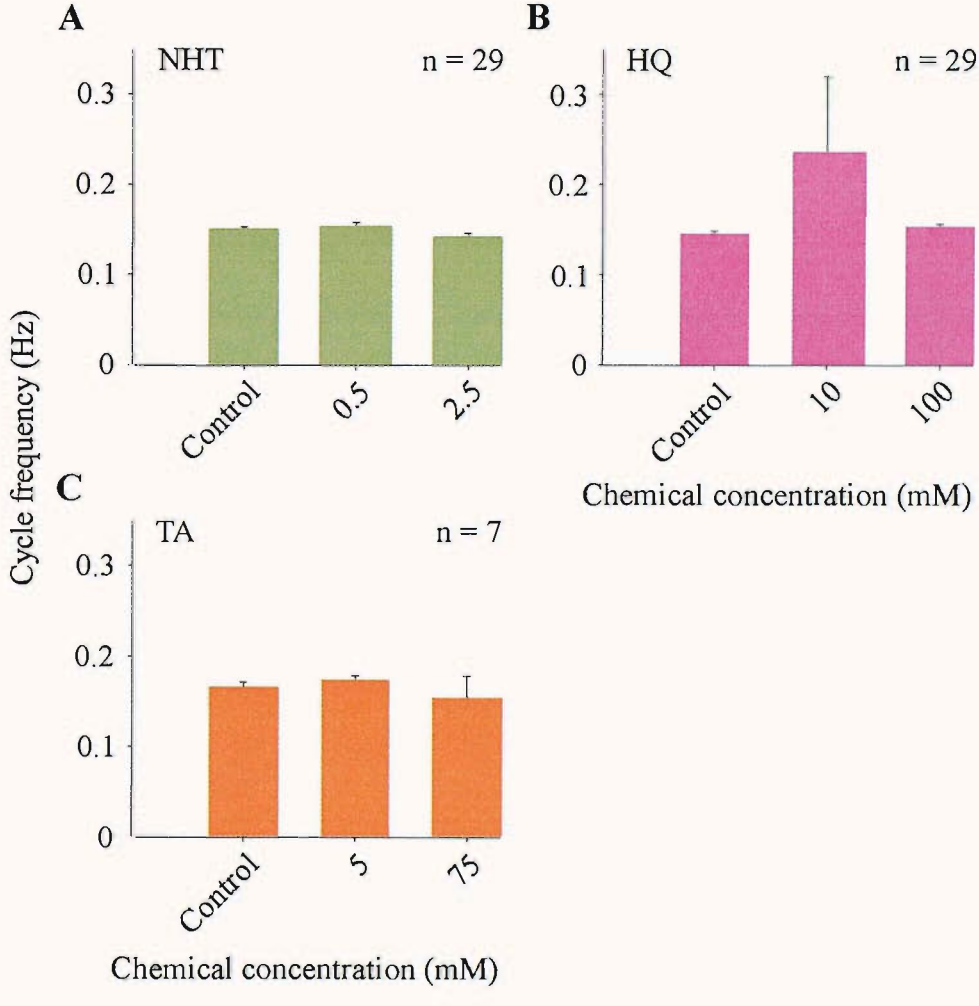



**Figure 4.9. The effect of the anti-feedants NHT, HQ and TA on the cycle frequency of the digging rhythm. A.** Applying droplets of distilled water, 0.5mM and 2.5mM NHT to the ovipositor valves showed no statistically significant effect ($p > 0.05$) on the digging rhythm. **B.** Applications of the anti-feedant HQ to the ovipositor valves also showed no statistically significant effect on the cycle frequency of the digging rhythm. **C.** No concentration dependent effects of the anti-feedant TA on the cycle frequency were observed ($p > 0.05$).

Increasing the concentration of all chemicals that were applied to the ovipositor valves did not result in any statistically significant effect on the cycle frequency of the digging rhythm. For all chemicals tested, there was no statistically significant difference in cycle frequency after chemical stimulation, between low and high chemical concentrations (Fig. 4.8, 4.9).

These results show that chemical stimulation of the ovipositor valves modifies the motor output of the CPG that underlies the oviposition digging rhythm in a concentration dependent manner by increasing the duration of cessation of the rhythm, but not the cycle frequency of the digging rhythm.

## 4.4 Discussion

It has been shown that all chemicals applied to the ventral surface of the ovipositor valves resulted in a cessation of the oviposition digging rhythm, and that the duration for which the rhythm stopped depended on chemical type and concentration. All chemicals at their higher respective concentrations resulted in statistically significant longer durations of cessation compared to their lower concentrations and to distilled water controls. No statistically significant concentration dependent effects of chemical stimulation of the ovipositor valves were observed on the cycle frequency of the digging rhythm on its return following cessation.

### 4.4a Contact chemosensory input and CPG's

Few studies have analysed the effects of chemosensory input on invertebrate CPG networks. In the pond snail *Lymnaea stagnalis*, chemical stimulation of the mouthparts with 100mM sucrose initiates rhythmic movements of the mouthparts (Kemenes *et al.* 1986). Experiments that have involved stimulating the mouthparts with lower concentrations of sucrose have established that a minimum concentration of 10mM sucrose is required to initiate feeding (Kemenes. pers. comm). To date however, the effects of stimulating the mouthparts with higher concentrations of sucrose are unknown. For example, it is unclear whether initiating feeding with 100mM sucrose and subsequently stimulating the mouthparts with 1 M sucrose would result in an increase in feeding frequency, or if 1M sucrose would act aversively and result in an inhibition of feeding. Further experiments are required in order to test these two possible outcomes. All sucrose stimulation of the ovipositor valves resulted in inhibition of the oviposition digging rhythm, although high (1 M) concentrations of sucrose, stopped the rhythm for significantly longer durations. In

contrast to experiments performed on *L. stagnalis*, no stimulatory effects of sucrose stimulation to the ovipositor valves were observed, for example, increases in cycle frequency of the digging rhythm upon stimulation of the ovipositor valves with 100mM sucrose.

In humans, contact chemosensory input, and therefore peripheral detection of chemicals, occurs in the oral cavity (Toubeau *et al.* 1994). When sugar based gels were treated with the bitter tasting chemical quinine, a similar concentration dependent effect was found on the number of rhythmic chewing cycles for different concentrations of quinine (Neyraud *et al.* 2005). More rhythmic chewing cycles were observed for low concentrations of quinine treated gels and untreated gels. As the quinine concentration of the gel was increased, the number of chewing cycles significantly decreased (Neyraud *et al.* 2005). In this study, although the digging rhythm stopped immediately after chemical stimulation of the valves, the duration from chemical stimulation (digging rhythm stopping) to re-activation of the digging rhythm was significantly longer for high concentrations of all chemicals, compared to low chemical concentrations and distilled water controls. In addition, in locusts, on return of the digging rhythm following chemical stimulation the cycle frequency of the ovipositor valves showed no significant difference compared to that of distilled water controls. Similarly in humans, no effect of quinine concentration in the gels, was found on the chewing frequency, again showing similarities with the effects of hydroquinone on the cycle frequency of the oviposition digging rhythm on its return after chemical stimulation (Neyraud *et al.* 2005).

### 4.4b Contact chemosensory input and motor output of a neural network

The results of this study also show similarities to those of Rogers and Newland (2000), who showed that chemical stimulation of the hind legs resulted in the locust withdrawing its leg from an aversive chemical stimulus, as discussed previously (see *Chapter 2*). As chemical concentration increases locusts are increasingly likely to move to their leg away from the stimulus, with higher chemical concentrations acting more aversively than lower concentrations. The concentration at which a chemical acted aversively depended on chemical type, with dietary requirements acting at higher concentrations compared to that of anti-feedants (Rogers and Newland, 2000). For oviposition, high concentrations of all chemicals were more aversive than low concentrations, and modified motor output by increasing the duration of cessation of the rhythm. Again, the concentration at which chemicals were most aversive varied with chemical type.

The results of this study therefore suggest that basiconic sensilla located on the ovipositor valves provide chemosensory input to the CPG that underlies digging. As contact chemical stimulation influences motor output of the oviposition digging CPG, neurons or groups of neurons that process contact chemosensory input may simply gate the pattern generating network, since it appears not to affect the rhythm itself. Intracellular recordings of spiking local interneurons in the metathoracic ganglion have been shown to respond to contact chemosensory stimulation (Rogers and Newland, 2002) in a concentration dependent manner. For example, 100mM NaCl evoked significantly more action potentials in a spiking local interneuron than 25mM NaCl and distilled water controls. It is possible therefore that spiking local interneurons may be part of the neural network that underlies oviposition digging. Indeed, Tousson (2001) showed that spiking local interneurons in the terminal abdominal ganglion respond to chemical stimulation of the ovipositor valves.

In summary, high concentrations of all chemicals stopped the oviposition digging rhythm for significantly longer periods compared to their lower concentrations and distilled water controls. Chemical stimulation of the ovipositor valves had no effect on the cycle frequency on return of the oviposition digging rhythm after cessation. The CPG that underlies oviposition digging is partly located within the terminal abdominal ganglion of the ventral nerve cord (Thompson, 1986a). As contact chemosensory input modifies motor output of the CPG, this raises the possibility that motor output is modified by chemosensory input at the level of the terminal abdominal ganglion of the central nervous system.

# Chapter 5 – Modulation of the oviposition digging rhythm

Chapter 5

# Modulation of the oviposition rhythm

## 5.1 Introduction

The behaviour of animals needs to be shaped to suit the demands posed by their fluctuating external environments. Previous studies, in different model organisms, have shown that CPG's are not fixed, hard-wired neural circuits, but instead are flexible multifunctional systems capable of producing different motor outputs (Harris-Warrick and Marder, 1991). In particular we know much about the modulation of the CPGs underlying locomotion in vertebrates (Bussieres and Dubuc, 1992), flight in insects (Libersat, 1992; Weiseleichler and Libersat, 1996) and the initiation and modulation of rhythmic feeding movements of molluscs and crustaceans (Elphick *et al.* 1995; Vehovszky *et al.* 1998; Coleman *et al.* 1992; Tazaki, 1993). The output of a CPG can be modulated by sets of neurons that use different neurotransmitters and neuromodulators (Kyriakides and McCrohan, 1989) and have the potential to produce different or specific modes of CPG output (Zelenin *et al.* 2001). The action of a neuromodulator on a CPG that underlies a specific behaviour functions to temporarily modify motor, and therefore behavioural output, by modifying the synaptic connectivity between the different neuronal components of the CPG and by altering the membrane properties of individual or groups of neurons (Balaban, 2002).

### 5.1a Background on nitric oxide signalling

The ubiquitous free radical, NO serves as a key signalling molecule in the central nervous system (Garthwaite *et al.* 1988) and at low concentrations acts as a neuromodulator by diffusing from its site of synthesis in three dimensions

(Lancaster, 1994; Wood and Garthwaite, 1994) to potential targets throughout an animal. NO is synthesized from its substrate L-arginine in a $Ca^{2+}$/calmodulin-dependent process by the enzyme nitric oxide synthase (NOS), in a reaction requiring oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) (Moncada *et al.* 1991).

Although the identification of NO as the causal factor underlying smooth muscle relaxation in blood vessels was not realized until 1987 (Moncada *et al.* 1987), the presence of a smooth muscle relaxant in blood vessels, or endothelial derived relaxing factor (EDRF) had been known since 1980 (Furchgott and Zawadski, 1980). Experiments using strips of rabbit aorta showed conflicting results when bathed with acetylcholine. Some preparations showed muscle contraction (shortening of the aorta) and some preparations showed muscle relaxation (lengthening of the aorta) (Furchgott and Zawadski, 1980). It was later realized that a difference in the preparation of rabbit aorta prior to acetylcholine application accounted for these conflicting results. Aorta whose inner surface had been handled directly during preparation showed muscle contraction in the presence of acetylcholine. Conversely, aorta whose inner surface had not been handled showed muscle relaxation in the presence of acetylcholine, indicating that something had been removed from the inner surface of aorta during handling, subsequently identified as endothelial cells (Furchgott and Zawadski, 1980). The muscle relaxant effects of acetylcholine in aorta with endothelial cells present indicated that acetylcholine was instructing endothelial cells to produce a then unidentified relaxing factor.

Moncada *et al.* (1987) subsequently showed using comparative pharmacological techniques that EDRF and NO both inhibited the aggregation of blood cells (platelets) and moreover, that inhibition of platelet aggregation by EDRF

was accounted for by the amounts of NO produced and released by endothelial cells, thus identifying EDRF as NO (Moncada *et al.* 1987).

NO was subsequently found to be present in the mammalian brain when antibodies raised against the enzyme required for NO production, NOS, showed strong staining in areas such as the posterior pituitary gland, the autonomic nerve fibres of the retina, the adrenal medulla and the olfactory bulb (Bredt *et al.* 1990). NOS is also co-localised with NADPH and the presence of NOS can therefore be detected using NADPH-diaphorase (NADPHd) staining (Dawson *et al.* 1991). NOS was subsequently found in neurons that innervated the monkey and human digestive system (Degiorgio *et al.* 1994). Subsequent studies showed that NO influenced insulin secretion in the human pancreas (Coiro *et al.* 1997) and further demonstrated that NO was an important neuronal signalling molecule in vertebrates that had physiological consequences when synthesised in, and released by the nervous system (Toda and Okamura, 1990).

### 5.1b Molecular targets of NO

As a result of NO being identified as an important signalling molecule in the nervous system, many subsequent studies focused on the molecular targets of NO. NO can act on a range of molecular targets in order to produce physiological effects. The targets of NO can include a direct action on cGMP regulated phosphodiesterases (Takemoto *et al.* 1993), sodium and potassium ion channels (Hammarström and Gage, 1999; Hampl *et al.* 1995), and the enzyme soluble guanylate cyclase (sGC) (Bredt and Snyder, 1989). The subsequent targets of cGMP can include cyclic nucleotide gated ion channels (Ahmad *et al.* 1994), cGMP-dependent protein kinases (Clementi *et al.* 1995), and cGMP-regulated cyclic nucleotide phosphodiesterases

(Lincoln and Cornwell, 1993). The most common target of NO is the enzyme sGC, which results in the production cGMP.

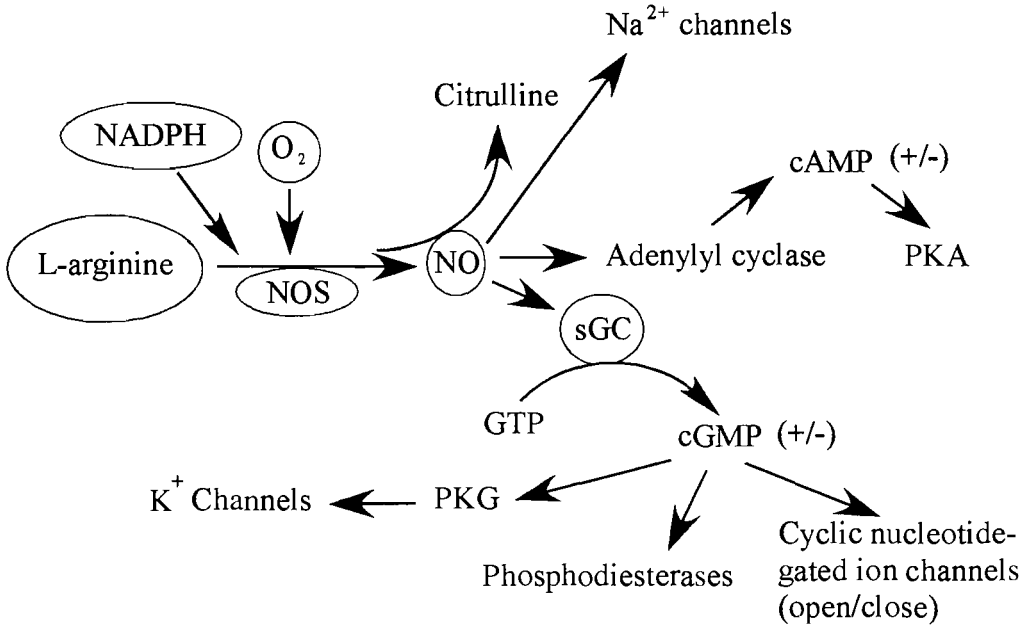



**Figure 5.1. The NO/cGMP signalling pathway**. The most common target of NO is sGC, which can subsequently elevate intracellular cGMP. cGMP in turn, can act to open or close cyclic nucleotide-gated ion channels. sGC can increase or decrease intracellular cGMP levels. cGMP can target cGMP-dependent phosphodiesterases or cGMP- dependent protein kinase (PKG). An alternative target for NO is adenylyl cyclase which can increase or decrease cAMP levels which in turn can target cAMP-dependent protein kinase (PKA). NO can also act directly on sodium channels, or on potassium channels via PKG.

cGMP may in turn act upon cGMP-dependent protein kinase (PKG). PKG is thought to phosphorylate downstream target proteins or affect the opening and closing of potassium channels that regulate neuron responses (Bredt and Snyder, 1989; Hampl *et al.* 1995). Alternatively, NO may target adenylyl cyclase which activates cAMP which in turn activates PKA (Fig. 5.1).

### 5.1c NO in vertebrate nervous systems

In vertebrates, the physiological actions of NO have been detected in the parasympathetic nervous system that controls renal, hepatic and digestive system

function, where its actions are vasodilatory (Baylis *et al.* 1990). The physiological consequence of the presence of NO in these systems is ion and nutrient re-uptake as well as osmoregulation (Hubschle *et al.* 1999). The presence of NOS has also been confirmed in the taste organ of the channel catfish, *Ictalurus punctatus*, using NADPHd staining. Although to date no behavioural studies have confirmed the function of NO in the taste organ of *I. punctatus*, its presence suggests a potential role in the peripheral detection of chemicals (Huque and Brand, 1994).

The presence of NO in the central nervous system of vertebrates has also been detected and its functions range from modulatory to toxic (Cudeiro and Rivadulla, 1999). In the spinal cord of frog larvae, *Xenopus laevis* (McLean and Sillar, 2004), the presence of the NO donor SNAP significantly increases the duration between bursts of motor activity. Conversely, when the NO scavenger, PTIO was applied to the ventral root of the spinal cord, the period between bursts of motor activity decreased, demonstrating that NO modulates the motor output of swimming movements in *Xenopus* (McLean and Sillar, 2004).

In myelin producing cells called oligodendrocytes, NO derived from macrophages has been found to contribute to demyelinating diseases such as multiple sclerosis (Mitrovic *et al.* 1994). The NO donor SNAP, at low concentrations inhibits mitochondrial respiration, but does not kill oligodendrocytes in cultured rat tissue. Increasing the concentration of SNAP however results in significant cell death (Mitrovic *et al.* 1995). These results suggest that the modulatory or toxic action of NO depends on its concentration. Moreover, NO levels are known to increase in response to certain physiological changes in the hepatic system. For example, acetaminophen, an analgesic that induces hepatic necrosis was found to produce more NO than untreated control animals (Gardner *et al.* 1998).

### 5.1d NO and invertebrate nervous systems

Anatomically, NOS-containing neurons have been detected throughout the central nervous system in various invertebrates by using NADPHd histochemistry. Neurons that contain NOS have been identified in the central nervous system of molluscs (Moroz *et al.* 1992; Elofsson *et al.* 1993), insects (Ott and Burrows, 1999; Ott *et al.* 2001; Ott and Elphick, 2002) and crustaceans (Schuppe *et al.* 2001). In locusts, stained clusters of neurons containing NOS are localised in varying densities in all 11 ganglia located along the entire length of the ventral nerve cord (Ott *et al.* 2001). As a result, NO has been hypothesized to play a crucial role in the modulation of sensory input in locusts (Elphick *et al.* 1996; Ott *et al.* 2001). In the visual system increasing NO levels in the optic lobe has been shown to affect the amplitude of corneal electroretinogram (ERG) measurements in response to light (Schmachtenberg and Bicker, 1999). Conversely, decreasing NO levels decreased the amplitude of the ERG amplitude in response to the same levels of light (Schmachtenberg and Bicker, 1999). This has lead to suggestions that increased NO within the optic lobe functions as a gain control mechanism and alters the sensitivity of the photoreceptors to increased or decreased light levels (Jones and Elphick, 1999).

As in other invertebrates such as molluscs, NO has been shown to have a modulatory role in the processing of olfactory stimuli in insects (Nighorn *et al.* 1998). Odorant-evoked nitric oxide signals are also observed in the antennal lobe of the hawk moth, *Manduca sexta,* in response to olfactory stimuli (Daly *et al.* 2001). Different odorants evoke spatially distinct patterns of NO production from neurons located in the antennal lobe (Collmann *et al.* 2004). NOS containing interneurons have been identified in the antennal lobes of locusts (Elphick *et al.* 1995), where neural oscillations are also observed in response to olfactory stimuli (Laurent *et al.*

1999). There are, to date, no studies that show spatially distinct NO production when locusts are exposed to different odours. Evidence from *M. sexta* suggests that similar, spatially distinct odorant-evoked NO signals may be occurring in the antennal lobes of locusts in response to olfactory stimuli.

Recent studies have implicated NO in modulating contact chemoreception and feeding behaviours. The neuronal networks that underlie the feeding movements in locusts are located in the suboesophageal ganglion (Schachtner and Braunig, 1993). Bath application of the NO donor, sodium nitroprusside (SNP), to the sub-oesophageal ganglion initiates feeding movements (Rast, 2001), an effect that can be reversed by bath application of the sGC inhibitor, ODQ. This suggests NO acts directly upon its common target sGC in order to initiate feeding.

Recent studies have also shown that NO is involved in the modulation of sensory input. The presence of NO has been demonstrated using NADPHd staining techniques in insects (Ott and Burrows, 1999; Ott *et al.* 2001; Ott and Elphick, 2002) and vertebrates (Porteros *et al.* 1996; Artero *et al.* 1995). Moreover, intense NADPHd staining of the antennal lobes in insects (Müller, 1994), and the taste buds of vertebrates (Huque and Brand, 1994; Kretz *et al.* 1998) suggest that NO may have a role in the modulation of olfactory and contact chemosensory input respectively. My experiments (see Chapter 4) have shown that contact chemosensory stimulation of the ovipositor valves modifies the motor output of the oviposition rhythm. Moreover, previous studies have also shown that NO can activate the CPG that underlies feeding in molluscs and insects (Elphick *et al.* 1995; Rast, 2001). This raises the possibility that NO may modulate the motor output of the oviposition rhythm.

This chapter provides a detailed analysis of the role of NO in the modulation of the oviposition rhythm. The levels of NO in the terminal abdominal ganglion

were manipulated by the bath application of pharmacological agents. The effect of these pharmacological agents on the cycle frequency of the digging rhythm are discussed.

## 5.2 Materials and Methods

### 5.2a Locusts

Female desert locusts, *Schistocerca gregaria* (Forskål) maintained and reared as described in *Chapter 2*.

### 5.2b Physiological methods

Recordings of the muscles were obtained as previously described in *Chapter 3*, although muscle activity was recorded primarily from the ventral opener muscles due to their large size and their accessibility. Cycle frequency was determined from the 1$^{st}$ spike of each muscle burst. After each experiment the animal was dissected and the locations of the wires visually confirmed.

### 5.2c Drug application

To apply drugs to the terminal abdominal ganglion, a small window of cuticle was removed from the ventral surface of sub-genital plate (Fig. 5.2). All pharmacological agents were obtained from Sigma Aldrich Chemical Co., Ltd and Tocris Cookson, Ltd., including L-arginine (L-arg), N-nitro-L-arginine methyl ester (L-NAME), N-nitro-D-arginine methyl ester (D-NAME), S-nitroso-N-acetyl-penicillamine (SNAP), N-acetyl-penicillamine (NAP), NOC-15; 3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANONOate), 8-Bromoguanosine 3': 5'-cyclic monophosphate (8-Br-cGMP), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) and (9*S*, 10*R*, 12*R*)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9, 12-epoxy-1*H*-di indolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT-5823).

Each chemical was maintained in the dark and only dissolved in normal locust saline (except ODQ) to the required concentration immediately prior (3 min) to bath application, with the exception of de-gassed SNAP which was maintained in the dark at room temperature for approximately 24 hours prior to use. Both ODQ and KT-5823 were first dissolved in 100% ethanol and then serially diluted to a final concentration of 0.1% ethanol. The exposed terminal abdominal ganglion was constantly perfused with fresh locust saline using a 502S Watson-Marlow microtube pump. Ganglia were exposed to a drug for 10 min and then washed with locust saline for 30 min. Each animal was tested only once per drug application.

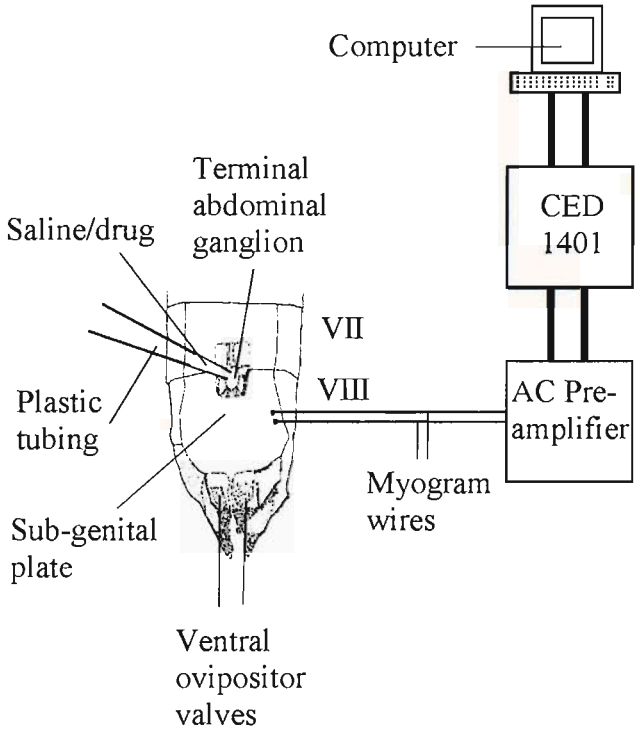



**Figure 5.2. Experimental set up for the delivery of drugs to the terminal abdominal ganglion.** Saline and drugs were delivered to the terminal abdominal ganglion via thin plastic tubing. Muscle activity was recorded using myogram wires inserted into the muscle under investigation, the signals from which were amplified with an AC pre-amplifier. Signals were then digitised using CED 1401 software and analysed using CED Spike 2 v4.0 software.

## 5.3 Results

To determine if NO acts to modulate the motor output of the oviposition digging rhythm, endogenous and exogenous NO levels were manipulated within the terminal abdominal ganglion by applying a variety of pharmacological agents. Their effects on the motor output of the CPG that underlies the oviposition digging movements were then analysed.

### 5.3a Effects of increasing endogenous and exogenous NO levels on the digging rhythm

To elevate endogenous levels of NO, the substrate for its synthesis, L-arginine, was bath applied at a concentration of 20mM to the terminal abdominal ganglion (Fig. 5.3). This produced a significant increase in the cycle frequency of the digging rhythm, as determined from myogram recordings from the opener muscles, from 0.13 ± 0.0004 Hz to 0.14 ± 0.0004 Hz (mean ± S.E.M, n = 8 animals, Students T-test; $t = 2.44$, $p < 0.05$). Following a 30 min wash in fresh saline, the cycle frequency returned to control (0.12 ± 0.0004 Hz) and was not significantly different from initial control levels (Fig. 5.3A, B).

Exogenous levels of NO were increased within the terminal abdominal ganglion by bath application of the NO donors SNAP and PAPANONOate, both at a concentration of 0.2mM. Increases in the level of exogenous NO using SNAP produced a significant increase in the cycle frequency of the digging rhythm, from a control value of 0.17 ± 0.0007 Hz to 0.21 ± 0.012 Hz (mean ± S.E.M, n = 5 animals, Students T-test; $t = 2.5$, $p < 0.05$) (Fig. 5.3C, D). A 30 min wash with normal locust saline again resulted in a recovery of the digging rhythm to a cycle frequency (0.19 ± 0.0008 Hz), which was not significantly different from the original control value.

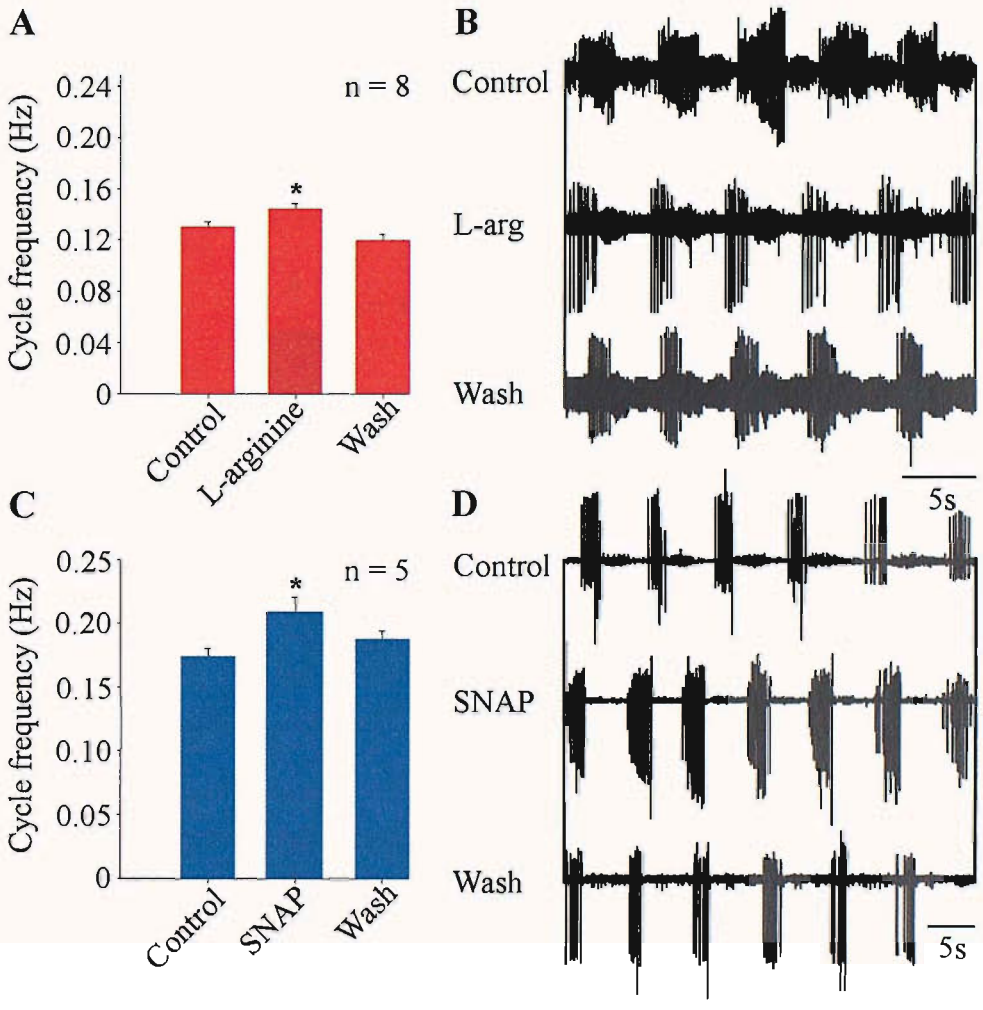



**Figure 5.3. The effect of NO on the cycle frequency of the digging rhythm. A.** The application of 20mM L-arginine caused a statistically significant increase in cycle frequency ($t = 2.44$, $p < 0.05$). Mean ± S.E.M. from 8 animals. **B.** An example showing the effect of a 10 min bath application of 20mM L-arginine on ovipositor valve opener muscles. The number of cycles of muscle activity increased from a control value of 5 to 6 after L-arginine application within the same time period. After a 30 min wash with locust saline, the number of cycles of opener muscle activity returned to a control value of 5. **C.** A 10 min bath application of SNAP resulted in a statistically significant increase in the cycle frequency of the digging rhythm ($t = 2.5$, $p < 0.05$). Mean ± S.E.M. of 5 animals. **D.** The effect of increased exogenous levels of NO on the digging rhythm. A 10 min bath application of 0.2mM SNAP resulted in an increase in the numbers of cycles of muscle activity from a control value of 6 to 7. A 30 min wash with locust saline resulted in the number of cycles of muscle activity returning to control (* = $p < 0.05$).

Similarly, increasing exogenous levels of NO by bath application of 0.2mM PAPANONOate (Fig. 5.4) also resulted in a statistically significant increase in the cycle frequency of the rhythm, from a control value of 0.16 ± 0.007 Hz to 0.24 ±

0.03 Hz (mean ± S.E.M, n = 5 animals, Students T-test; t = -2.82, $p < 0.05$). A 10 min wash with locust saline resulted in the cycle frequency of the rhythm returning close to its control value (0.17 ± 0.007 Hz) (Fig. 5.4A, B).

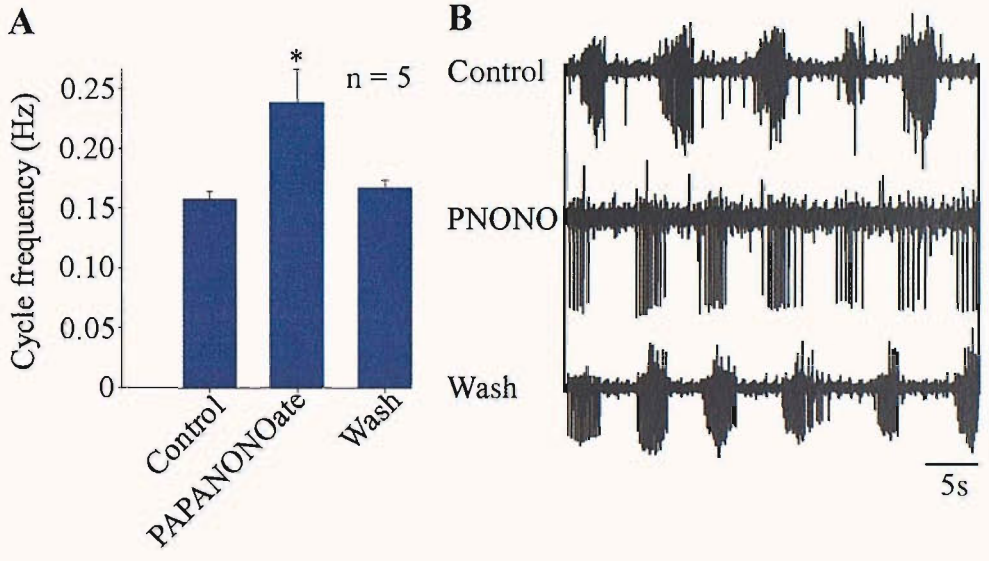



**Figure 5.4. The effect of the NO donor, PAPANONOate (PNONO), on the cycle frequency of the digging rhythm. A.** A 10 min bath application of 0.2mM PAPANONOate caused a statistically significant increase in the cycle frequency (t = -2.82, $p < 0.01$) (* = $p < 0.05$). The data presented here is the mean and S.E.M. of 5 animals. **B.** A 10 min bath application of 0.2mM PAPANONOate resulted in a significant increase in the number of cycles of muscle activity within the same time period, from a control period of 5, to a test value of 7. A 30 min wash with locust saline resulted in the digging rhythm partially returning to its original cycle frequency.

### 5.3b Effects of reducing NO levels on the oviposition digging rhythm

To decrease the availability of endogenous NO, a NOS inhibitor, L-NAME, was bath applied (Fig. 5.5). 20mM L-NAME produced a significant decrease in the cycle frequency of opener muscle activity from 0.15 ± 0.0006 Hz to 0.13 ± 0.0006 Hz (mean ± S.E.M, n = 8 animals, Students T-test; t = 2.08, $p < 0.05$). Following a 30 min wash with normal locust saline the digging rhythm showed a partial recovery to its original control value (0.15 ± 0.012 Hz), which was not significantly different from the initial control (Fig. 5.5A, B).

To confirm the specificity of the action of L-NAME, D-NAME was also applied. A 10 min bath application of the isomer D-NAME, had no effect upon the cycle frequency of the digging rhythm (control = 0.15 ± 0.0007 Hz, test = 0.15 ± 0.0008 Hz, mean ± S.E.M, n = 4 animals, Students T-test; t = 0.12, $p > 0.05$) (Fig. 5.5C, D).

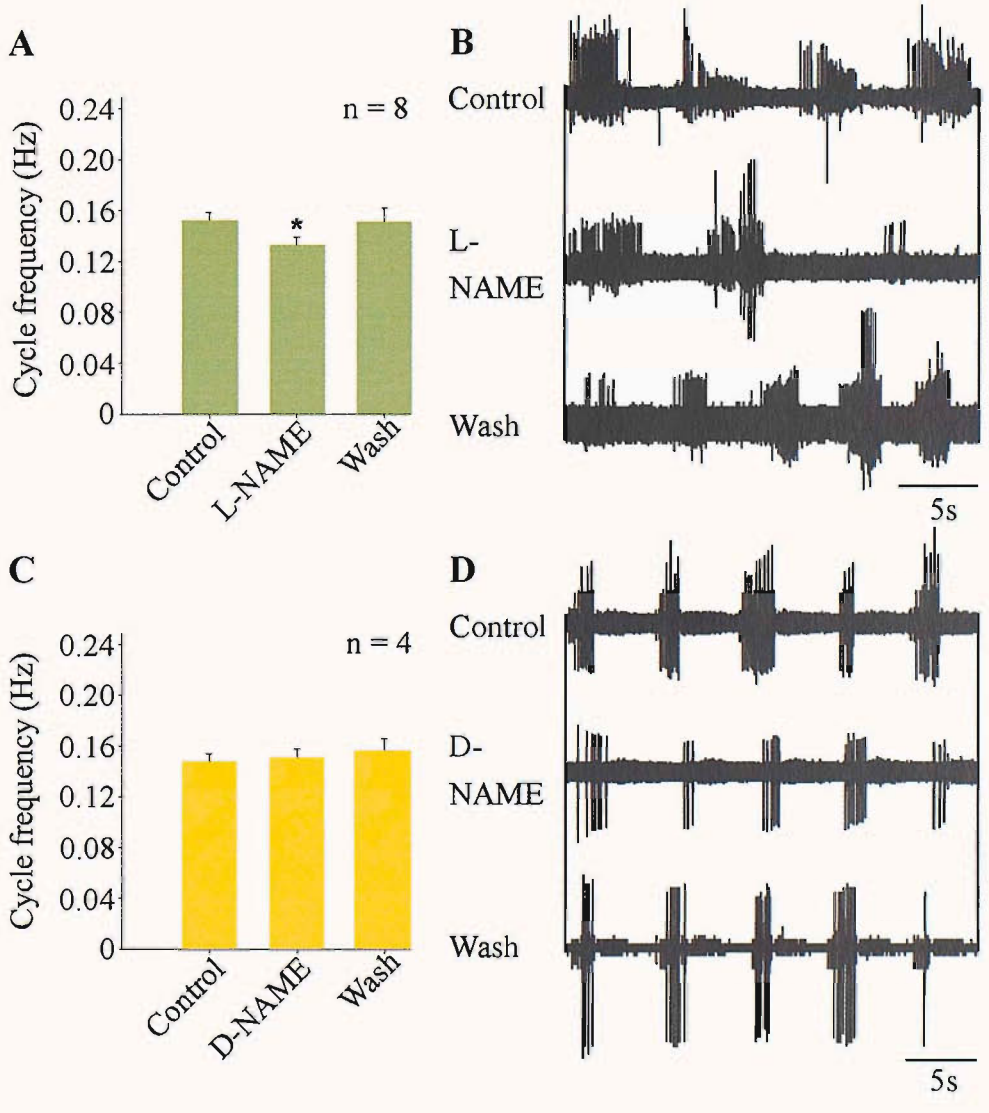



**Figure 5.5. The effect of inhibiting NO synthesis by bath application of the NOS inhibitor L-NAME. A.** A 10 min bath application of 20mM L-NAME caused a statistically significant decrease in the cycle frequency of the digging rhythm (t = 2.08, $p < 0.05$). Means ± S.E.M. from 8 animals. **B.** A 10 min bath application of L-NAME resulted in a decrease in the number of cycles from a control value of 4, to a test value of 3. The effects of L-NAME were reversed by a 30 min wash with locust saline. **C.** A 10 min bath application of the inactive isomer 20mM D-NAME caused no statistically significant change in cycle frequency (t = 0.12, $p > 0.05$). Means ± S.E.M from 4 animals. **D.** A 10 min bath application of 20mM D-NAME resulted in no significant decrease in the number of cycles between test and control values (* = $p < 0.05$).

Decreasing endogenous NO levels was also achieved by bath applying the NO scavenger PTIO at a concentration of 0.5mM (Fig. 5.6A, B). This resulted in a significant decrease in the cycle frequency of the digging rhythm from a control value of 0.17 ± 0.001 Hz to 0.14 ± 0.0005 Hz (mean ± S.E.M, n = 11 animals, Students T-test, t = -2.04, $p < 0.05$). A 30 min wash with locust saline resulted in a return of the cycle frequency to a level that was not significantly different from its original control value (0.18 ± 0.001 Hz) (Fig. 5.6A, B).

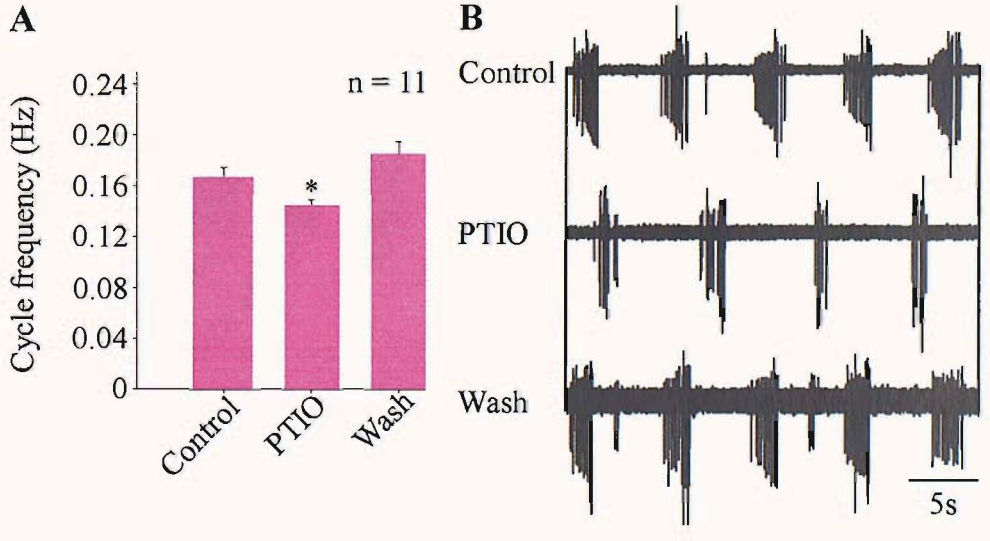



**Figure 5.6. The effect of removing endogenous NO by bath application of the NO scavenger PTIO. A.** A 10 min bath application of 0.5mM PTIO caused a statistically significant decrease in cycle frequency (t = 2.04, $p < 0.05$). A 30 min wash with locust saline resulted in the digging rhythm returning to its original cycle frequency. Mean ± S.E.M. of 11 animals. **B.** An example showing that a 10 min bath application of 0.5mM PTIO resulted in a decrease in the number of cycles within the same time period, from a control value of 5 to a test value of 4 (PTIO) (* = $p < 0.05$).

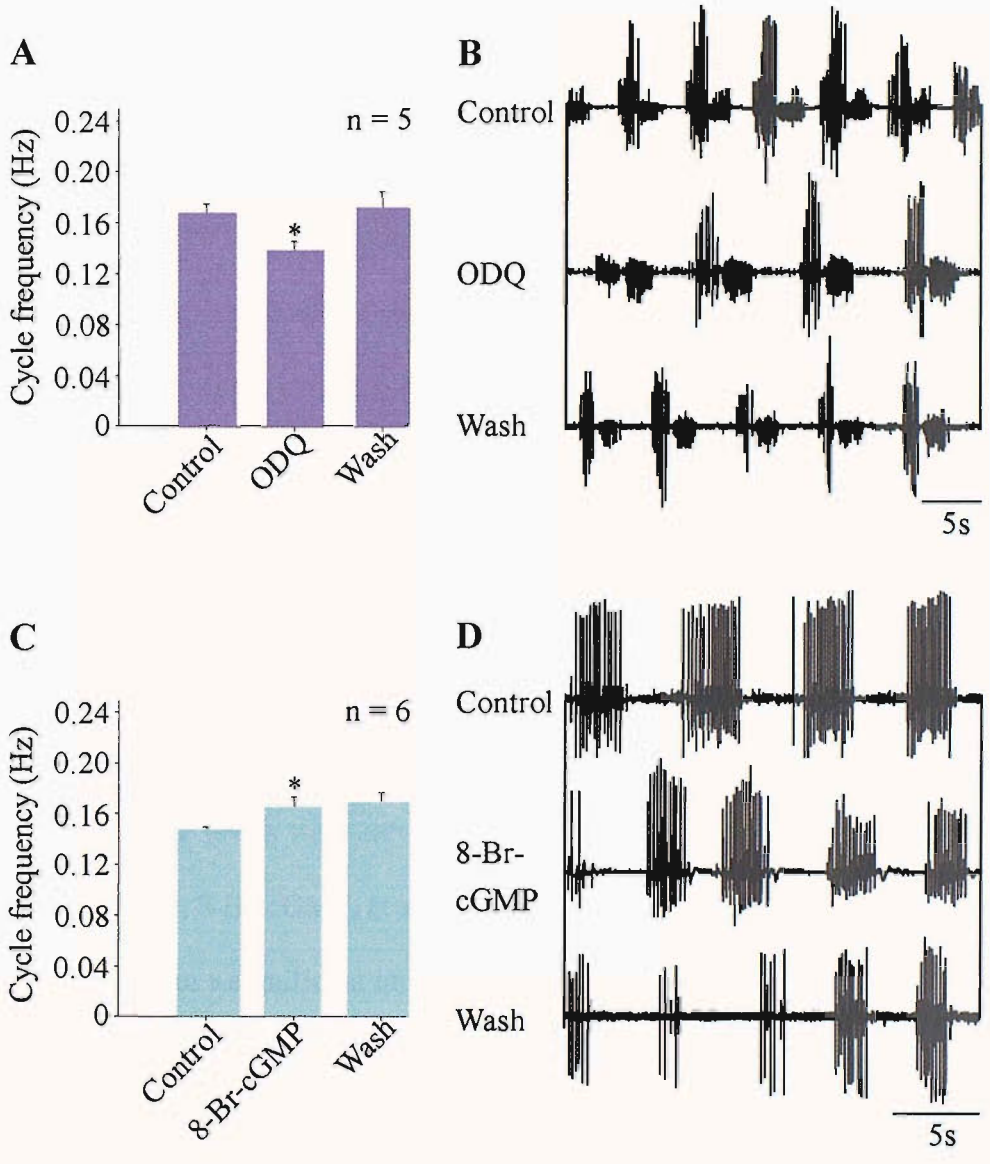



**Figure 5.7. The effects of the sGC inhibitor, ODQ and the cGMP analogue, 8-Br-cGMP on the digging rhythm. A.** A 10 min bath application of 0.1mM ODQ resulted in a significant decrease in cycle frequency ($t = 2.85$, $p < 0.05$). Means ± S.E.M. from 5 animals. **B.** A 10 min bath application of ODQ resulted in a decrease in the cycle number from a control of 6 cycles to a test value of 4 cycles within the same time period. After a 30 min wash with locust saline, the number of cycles increased to 5. **C.** A 10 min bath application of 0.1mM 8-Br-cGMP resulted in a significant increase in cycle frequency of the digging rhythm ($t = 2.22$, $p < 0.05$). A 30 min wash with locust saline did not result in the cycle frequency returning back to its original value. Means ± S.E.M. from 6 animals. **D.** Bath application of 8-Br-cGMP resulted in an increase in the number of cycles of muscle activity of the digging rhythm, from a control value of 4 cycles to a test value of 5 within the same time period (* = $p < 0.05$).

### 5.3c The modulatory effect of NO on the digging rhythm is mediated via a sGC/cGMP signalling pathway

sGC acts as one molecular target of NO (Bredt and Snyder, 1989). To determine whether NO acts to modulate the oviposition digging rhythm via the sGC/cGMP signalling pathway, a specific inhibitor of sGC, ODQ, was bath applied to the terminal abdominal ganglion (Fig. 5.7). A 10 min bath application of 0.1mM ODQ resulted in a significant decrease in the cycle frequency of the digging rhythm from a control value of 0.17 ± 0.0007 Hz to a value of 0.14 ± 0.0007 Hz (mean ± S.E.M, n = 5 animals, Students T-test; t = 2.85, $p < 0.05$). A 30 min wash with locust saline resulted in a return of the digging rhythm to a frequency (0.17 ± 0.013 Hz) that was not significantly different to the original control value (Fig. 5.7A, B).

Cyclic GMP levels were elevated by bath applying a membrane permeable analogue of cGMP, 8-Br-cGMP, at a concentration of 0.1mM. Increasing the level of cGMP resulted in a significant increase in the cycle frequency of the digging rhythm, from a control value of 0.15 ± 0.0004 Hz to 0.17 ± 0.0008 Hz (mean ± S.E.M, n = 6 animals, Students T-test, t = 2.22, $p < 0.05$). A 30 min wash, however, failed to reverse the effects of 8-Br-cGMP within the wash time window (0.17 ± 0.0008 Hz) (Fig. 5.7C, D).

To demonstrate that the L-arginine-dependent effects on cycle frequency were due to increased NO synthesis acting via sGC, both L-arginine and ODQ were simultaneously bath applied to the terminal abdominal ganglion. This had the effect of elevating endogenous NO levels whilst simultaneously inhibiting sGC. Bath application of L-arginine alone significantly increased the frequency of the digging rhythm (see Fig. 5.3A, B). Simultaneous bath application of L-arginine and ODQ, however, had no significant effect on cycle frequency (n = 5 animals, Students T-test,

t = -1.34, $p > 0.05$) (Fig 5.8A, B). These results suggest that L-arginine acts to increase NO levels that then act to increase cycle frequency by activating sGC.

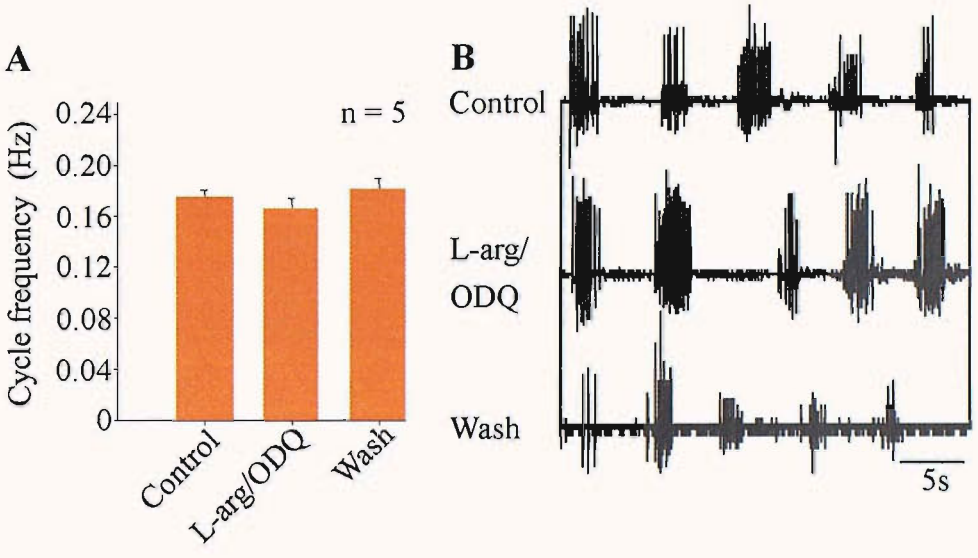



**Figure 5.8. The simultaneous bath application of the NO substrate L-arginine and the sGC inhibitor ODQ. A.** The simultaneous bath application of L-arginine and ODQ resulted in no statistically significant difference between control and test values (t = -1.34, $p > 0.05$). Mean ± S.E.M. are from 5 animals. **B.** Elevating endogenous NO levels by the bath application of 20mM L-arginine while simultaneously blocking the enzyme sGC via bath application of 0.1mM ODQ, resulted in no significant change in the number of cycles between control, test and wash values.

### 5.3d Does cGMP act via a protein kinase signalling pathway?

To determine whether NO acts to modulate the digging rhythm by acting on a downstream protein kinase, the generic protein kinase inhibitor, H-7 was bath applied to the terminal abdominal ganglion. Bath application of 0.1mM H-7 resulted in a significant decrease in the cycle frequency of the valve opener muscles from a control value of 0.19 ± 0.012 Hz to 0.094 ± 0.014 Hz (mean ± S.E.M, n = 5 animals, Students T-test, t = 4.93, $p < 0.05$). A 30 min wash with locust saline resulted in a partial recovery of the cycle frequency to 0.16 ± 0.014 Hz that was not significantly different from the original control value (Fig. 5.9A, B).

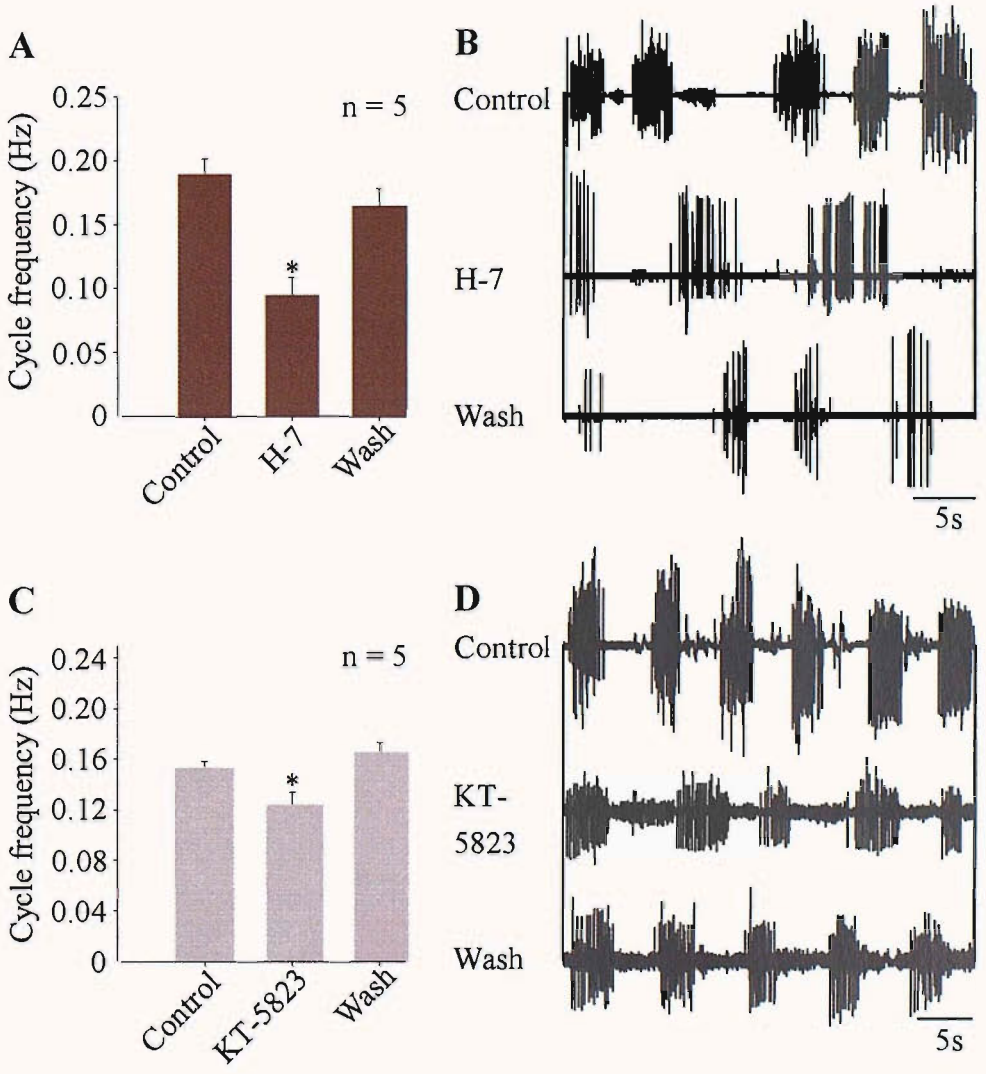



**Figure 5.9. The effect of the generic protein kinase inhibitor H-7 and the PKG inhibitor, KT-5823 on the digging rhythm. A.** A 10 min bath application of 0.1mM H-7 resulted in a highly significant decrease in cycle frequency ($t = 4.93$, $p < 0.05$). A 30 min wash with locust saline resulted in a partial recovery of the digging rhythm to control levels. Mean ± S.E.M. are from 5 animals. **B.** A 10 min bath application of H-7 resulted in a decrease in the number of cycles (3 cycles) compared to control value (5 cycles). A 30 min wash with locust saline resulted in a partial recovery of the digging rhythm to control levels. **C.** A 10 min bath application of 10μM KT-5823 resulted in a significant decrease in the cycle frequency ($t = 2.8$, $p < 0.05$). Mean ± S.E.M. are from 5 animals. **D.** A 10 min bath application of KT-5823 resulted in a decrease in the number of cycles (5 cycles) from a control value of 6. This recording shows that a 30 min wash with locust saline did not result in the number of cycles returning to its control value (* = $p < 0.05$).

To determine whether NO acts to modulate the digging rhythm by acting on PKG, the specific PKG inhibitor, KT-5823 was bath applied. Bath application of 10μM KT-5823 resulted in a significant decrease in the cycle frequency of the

digging rhythm from a control value of 0.15 ± 0.005 Hz to 0.12 ± 0.01 Hz (mean ± S.E.M. n = 5 animals, Students T-test, t = 2.8, $p < 0.01$). A 30 min wash with locust saline resulted in a recovery of the digging rhythm that was not significantly different from the original control value (Fig. 5.9C, D).

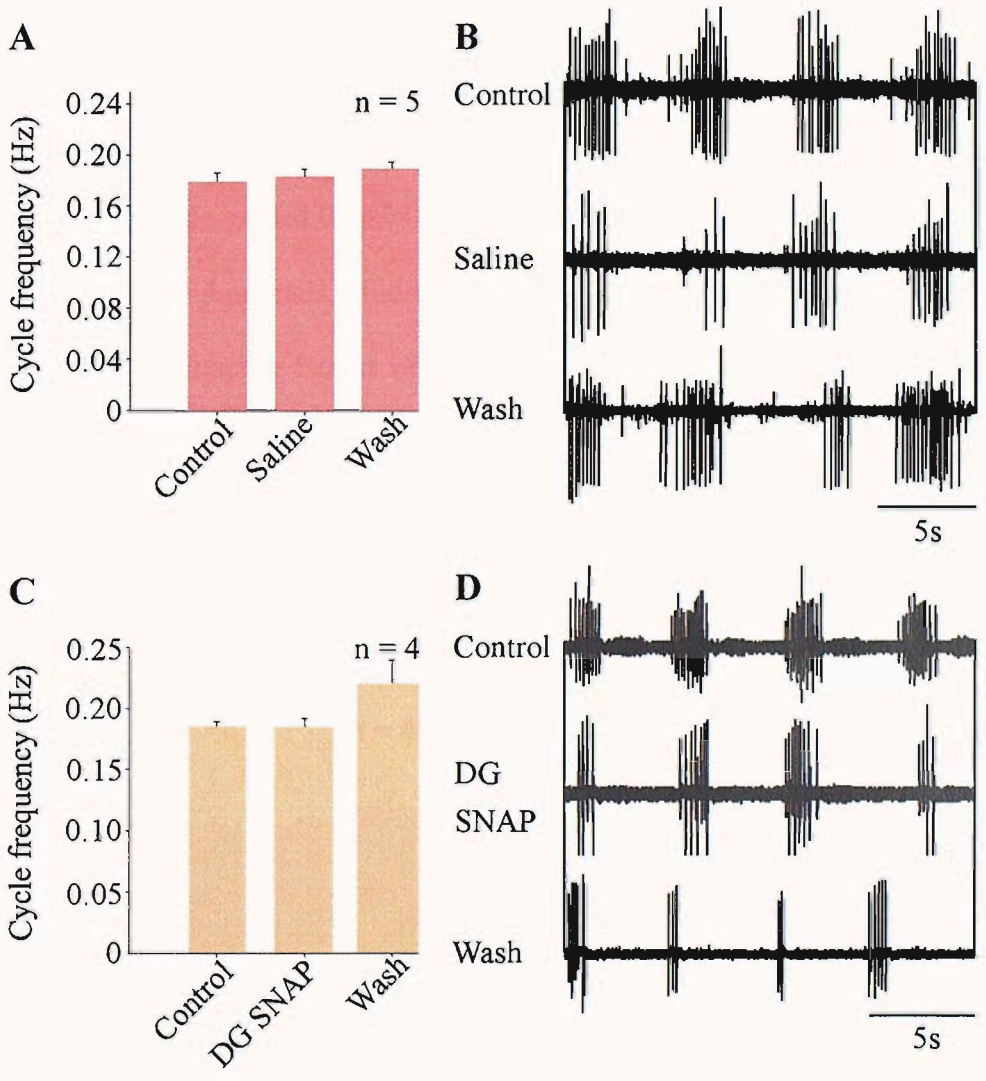



**Figure 5.10. Bath application of locust saline and de-gassed SNAP and its effect on the digging rhythm. A.** Bath application of locust saline resulted in no significant effect on cycle frequency (t = -1.81, $p > 0.05$). Means ± S.E.M. recorded from 4 animals. **B.** An example showing that bath application of locust saline for 10 min had no significant effect on the cycle frequency within the same time period. **C.** A 10 min bath application of 0.1mM de-gassed SNAP showed no significant effect on cycle frequency (t = 0.17, $p > 0.05$). The mean ± S.E.M. recorded from 4 animals. **D.** An example showing the effects of de-gassed SNAP on the cycle frequency of the digging rhythm. A 10 min bath application of de-gassed SNAP resulted in no significant difference in the number of cycles between control and test values.

To rule out the possibility that the pharmacological agents were modulating motor output by having non-specific effects, a series of further control experiments were performed. The long term bath application of locust saline for the entire duration of the experiment had no significant effect on cycle frequency (control = 0.17 ± 0.0008 Hz, test = 0.17 ± 0.00072 Hz, mean ± S.E.M, n = 4 animals, Students T-test; t = -1.81, $p > 0.05$) (Fig. 5.10A, B). SNAP that had been left to de-gas for 24 hours was also bath applied to the terminal abdominal ganglion. A 10 min bath application of de-gassed 0.1mM SNAP had no significant effect upon the digging rhythm (n = 4 animals, Students t-test, t = 0.17, $p > 0.05$) (Fig. 5.10C, D).

Finally, the inactive isomer of SNAP, NAP was bath applied for 10 min and no significant difference between control and test values was observed (n = 4 animals, Students T-test; t = 0.14, $p > 0.05$) (Fig. 5.11A, B).

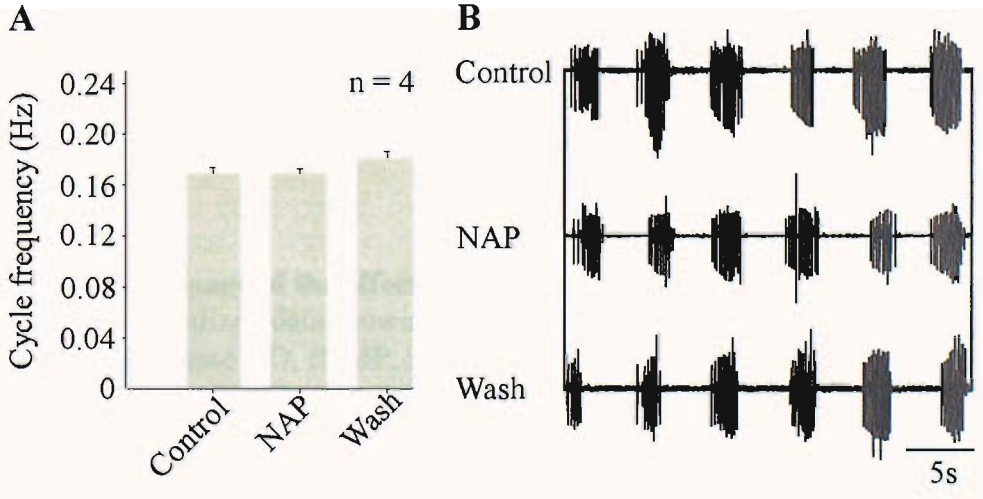



**Figure 5.11. The effect of bath application of the inactive isomer of SNAP, NAP, on the cycle frequency of the digging rhythm. A.** A 10 min bath application of 0.1mM NAP, had no effect on cycle frequency (t = 0.14, $p > 0.05$). The mean ± S.E.M. are from 4 animals. **B.** An example showing that a 10 min bath application of NAP resulted in no difference in the number of cycles between control and test values.

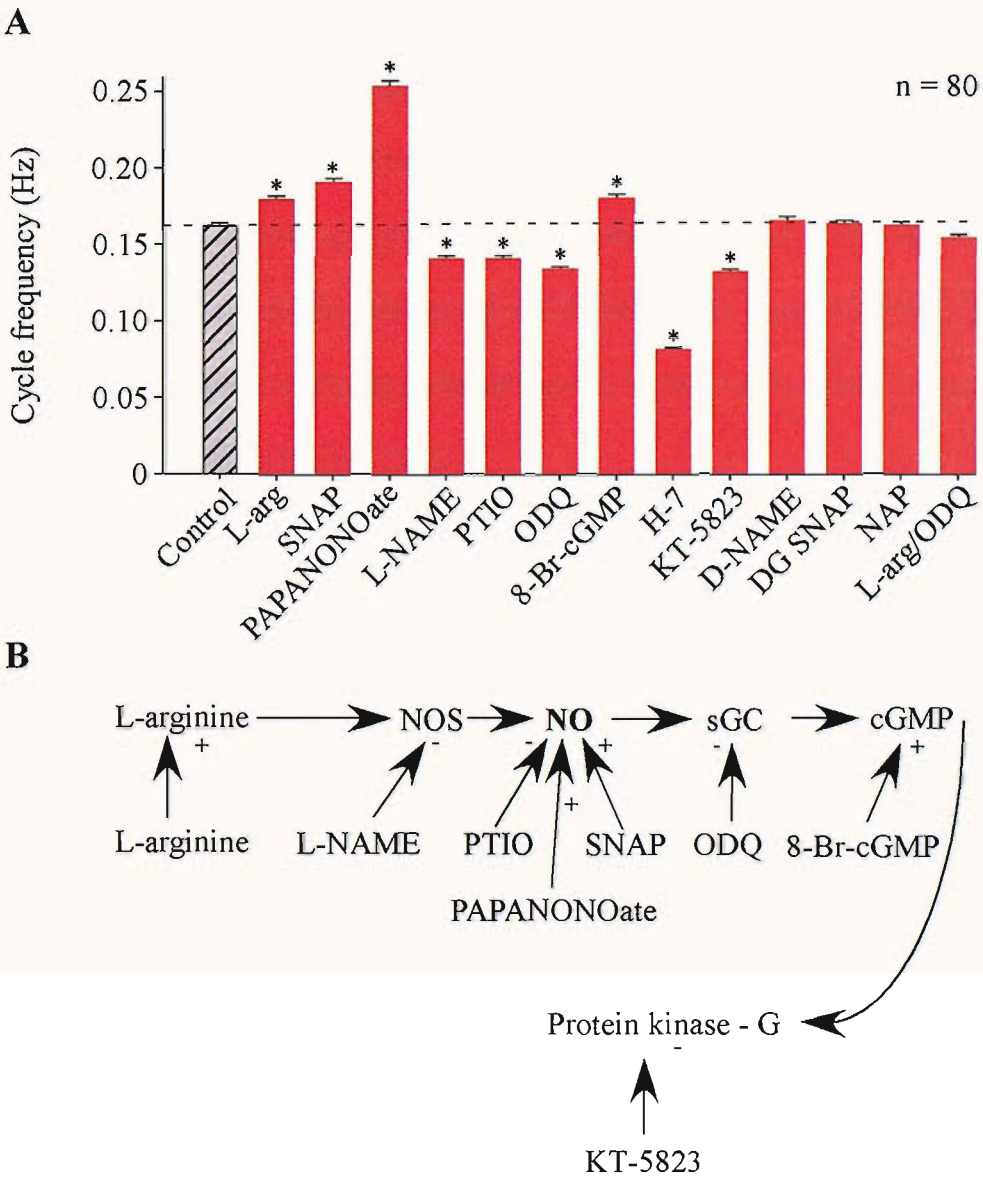



**Figure 5.12. Summary of the effects of NO on the cycle frequency of the digging rhythm. A.** Normalized data showing the means and S.E.M. for each of the drugs that elevate or decrease NO, cGMP, sGC and protein kinase, as well as the normalized control value (* = $p < 0.05$). **B.** A diagram of the NO/cGMP signalling pathway with the relevant drugs that affected NO presence and production. Plus and minus signs show where NO levels were elevated or decreased and where sGC and protein kinase were inhibited, and also where cGMP levels were elevated.

In summary, NO modulates the oviposition digging rhythm in locusts by acting via sGC to elevate cGMP which in turn acts via PKG. Figure 5.12A summarizes the effects of drugs that increase or decrease levels of NO. Also shown are the effects of the sGC inhibitor ODQ, the membrane permeable analogue of cGMP, 8-Br-cGMP and the generic protein kinase inhibitor and specific PKG

inhibitor, H-7 and KT-5823 respectively. Control data (D-NAME, de-gassed SNAP, NAP and the combined bath application of ODQ and L-arginine) are also shown for comparison. Figure 5.12B shows the NO/cGMP-PKG signalling pathway that has been established by bath applying the above mentioned drugs. The effects of those drugs on NO levels and the subsequent sGC/cGMP-PKG signalling pathway are denoted by a plus (+) or minus (-) to indicate an increase or decrease respectively in levels of NO, sGC, cGMP or PKG.

## 5.4 Discussion

This study has established that NO modulates the oviposition rhythm via the sGC/cGMP-PKG signalling pathway. These results show that increasing the availability of NO in the terminal abdominal ganglion of the locust, significantly increases the cycle frequency of the digging rhythm. Conversely, decreasing the availability of NO in the terminal abdominal ganglion resulted in a significant decrease in the cycle frequency. Inhibiting the enzyme sGC and increasing the availability of cGMP in the terminal abdominal ganglion, resulted in a significant decrease and increase in the cycle frequency of the digging rhythm respectively. Inhibiting a known target of cGMP, PKG, in the terminal abdominal ganglion, also resulted in a significant decrease in the cycle frequency of the oviposition digging rhythm.

### 5.4a The role of NO in modulating CPG's

In locusts, NO was found to initiate the CPG that underlies the rhythmic feeding movements of the mouthparts (Rast, 2001). When the NO donor SNP was bath applied to the suboesophageal ganglion that contains the CPG, the motor pattern underlying feeding was initiated (Rast, 2001). The effect of SNP was reversed (feeding inhibited) by bath application of L-NAME, and blocking sGC with ODQ resulted in an inhibition of the feeding motor pattern, suggesting that NO was acting via the molecular target, sGC. The downstream subsequent targets of cGMP were, however, not identified by Rast (2001). The results of this study go one step further than Rast (2001) and show that the CPG that underlies the oviposition digging rhythm is modulated via an NO/cGMP signalling pathway that acts via PKG.

NO also has a behavioural role in the chemosensory activation of feeding in the pond snail, *Lymnaea. stagnalis* (Elphick *et al.* 1995). In freely behaving *L.*

*stagnalis* stimulating the lips with sucrose initiates a feeding response consisting of a rhythmic tri-phasic protraction, rasp and swallowing movement of the mouthparts (Straub *et al.* 2002). The rhythmic feeding movements are produced by a CPG located in the buccal ganglion. Sucrose application to the mouthparts activates a fictive feeding rhythm in isolated lip and buccal ganglion preparations. Bath applying the NOS inhibitor L-NAME whilst stimulating the mouthparts with sucrose, however, resulted in an inhibition of the rhythm. NO application in the absence of sucrose, re-initiated the rhythm indicating that, as with the locust suboesophageal preparation, NO can act to initiate CPG activity (Elphick *et al.* 1995). Potential sources of NO within the buccal ganglion were identified using NADPH diaphorase staining which revealed staining in regions of the neuropil in which the median and superior lip nerves project (Elphick *et al.* 1995).

In *L. stagnalis*, feeding initiation depends on NO levels within the buccal ganglia that contain the feeding CPG (Elphick *et al.* 1995; Benjamin and Rose, 1979). Recent studies however, have indicated that the generation of NO in the buccal ganglion on initiation of feeding serves an inhibitory role, contradicting the findings of Elphick *et al.* (1995). The synthesis of NO upon feeding initiation has been shown to be produced by neurons that innervate the oesophagus (Sadamoto *et al.* 1998). Preventing NO synthesis and its effects on the feeding network, by lesioning a neuron that is part of the rhythmic oesophagus network (B2), leads to an increase in the frequency of the rhythmic feeding movements (Kobayashi *et al.* 2000). This result suggests that NO can have an inhibitory effect on the fictive feeding rhythm. This was subsequently confirmed by bath application of the exogenous NO scavenger, PTIO and the NOS inhibitor, L-NAME to the buccal ganglion that both resulted in a significant increase in the frequency of the fictive feeding rhythm. This has led to the hypothesis that NO modulates the feeding

rhythm in *L. stagnalis* by inhibiting CPG activity in preparation for the next cycle of feeding movements (Kobayashi *et al.* 2000). Differences in the methodology may however, have contributed to the contradictory findings between Elphick *et al.* (1995) and Kobayashi *et al.* (2000).

NO has also been shown to be involved in the modulation of vertebrate CPG networks. In the tadpole of the frog *Xenopus laevis*, increases in NO levels resulted in a significant decrease in the frequency of swimming movements (McLean and Sillar, 2004). In contrast to this study, an increase in NO increases the cycle frequency of the CPG underlying oviposition, why it should have this effect remains to be examined in the future. One possibility is that NO acts at the level of the synapse and modulates the release of neurotransmitter (Wildemann and Bicker, 1999). In Drosophila neuromuscular junction preparations, increasing NO levels at the synaptic bulb by bath applying the NO donor SNP resulted in a significant increase in the number of synaptic vesicles releasing neurotransmitter at the neuromuscular junction (Wildemann and Bicker, 1999). This study suggests that increased NO levels may act at the synapse to increase the amount of neurotransmitter released, either between the individual neuronal components of the CPG, or at the level of the neuromuscular junction to increase cycle frequency of the digging rhythm.

### 5.4b PKG signalling

Bath application of the generic protein kinase inhibitor, H-7 and the PKG inhibitor KT-5823 resulted in a modulation of the motor pattern by significantly decreasing the cycle frequency of the rhythm. It has been established that NO acts via a sGC/cGMP signalling pathway and that blocking protein kinase significantly decreases the cycle frequency of the digging rhythm. The effect of KT-5823

therefore demonstrates that the oviposition rhythm is modulated via a sGC/cGMP-PKG signalling pathway. PKG is known to modulate the behavioural responses to odours in both vertebrates and invertebrates, including insects (Shaver *et al.* 1998). In the nematode, *Caenorhabditis elegans*, a prolonged exposure to odours causes an adaptation to the behavioural response. *C. elegans* are known to exhibit positive chemotaxis (an attraction to certain chemicals) in the presence of certain chemical attractants including benzaldehyde, butanone and isoamyl alcohol. After continuous, repeated exposure to these odours, the attraction of *C. elegans* to these compounds decreases. Mutants of *C. elegans* unable to synthesize PKG, were unable to adapt to prolonged odour exposure (L'Etoille *et al.* 2002), showing that PKG is necessary for the behavioural adaptation to odours.

Although it has been established that NO can act via a cGMP/PKG signalling cascade, a common target of PKG is potassium channels (Hirsch and Schlatter, 1995). In the rat, the principal cell of the basolateral membrane of the cortical collecting duct of the kidney is potassium ($K^+$) conductive. Two $K^+$ channels have been described in the principal cell a small conductance $K^+$ and an intermediate conductance $K^+$channel (Costa and Assreuy, 2005). Activation of small conductance $K^+$ channels is blocked in the presence of the PKG inhibitor, KT-5823, indicating that PKG can act on small conductance $K^+$ channels. This suggests that neuronal events such as re-polarisation may be mediated by the presence of PKG via the phosphorylation of $K^+$ channels (Reddy, 2006). Inhibition of PKG using a specific inhibitor such as KT-5823, could therefore prevent, or decrease the rate of $K^+$ channel phosphorylation. This in turn could decrease the activity of $K^+$ channels and decrease the rate of re-polarisation of individual neuronal components of a CPG network and decrease the frequency of the oviposition digging rhythm. It remains to be determined exactly how PKG brings about the NO dependent modulation of

digging. It has however, been demonstrated for the first time that NO has a clear modulatory role in egg-laying behaviour.

# Chapter 6 – The role of nitric oxide in the modulation of contact chemosensory input

Chapter 6

# The role of nitric oxide in the modulation of contact chemosensory input

## 6.1 Introduction

An animal's behavioural response to sensory input needs to be modified in the context of its current internal physiological state (Elliott and Susswein, 2002). Although a range of neuromodulatory substances have been shown to affect animal behaviour (Harris-Warrick and Marder, 1991), NO has been shown to modulate behavioural responses to all of the known sensory modalities including mechanosensation (Schuppe and Newland, 2004), vision (Bicker and Schmachtenberg, 1997), audition (Zdanski *et al.* 1998; Hanson *et al.* 2003), olfaction (Gelperin *et al.* 1996; Nighorn *et al.* 1998; Bicker, 1998; Schmachtenberg and Bacigalupo, 1999; Collmann *et al.* 2004) and taste (Elphick *et al.* 1995; Kretz *et al.* 1998; Morley *et al.* 1997; Krizhanovsky *et al.* 2000; Kobayashi *et al.* 2000; Murata *et al.* 2004; Nakamura *et al.* 2005) in both vertebrates and invertebrates.

### 6.1a The role of NO in olfaction

In both vertebrates and invertebrates, NOS has been shown to be present in areas of the central and peripheral nervous system that are involved in the processing of olfactory input (Dellacorte *et al.* 1995; Huque and Brand, 1994; Elphick *et al.* 1995). In invertebrates, studies involving olfactory learning (Muller, 1996; Menzel *et al.* 1996), and conditioned taste aversion (Rabin, 1996; Prendergast *et al.* 1997) have all demonstrated the involvement of NO in modulating behavioural responses to olfactory input. For example, the slug, *Limax marginatus,* is capable of

discriminating between a number of different odours including octanol, hexonal and 3-methylcyclohexonal (Gelperin *et al.* 1996). An injection of the NOS inhibitor, L-NAME, into the area of the brain that processes olfactory stimuli (the protocerebral lobe) abolishes the ability of *L. marginatus* to discriminate between the odours (Sakura *et al.* 2004). Moreover, oscillations of neural activity within the protocerebrum occur in response to olfactory stimulation in molluscs (Toda *et al.* 2000). The odours of preferred foods increased the frequency of neural oscillations whereas aversive odours decreased the frequency of neural oscillation (Toda *et al.* 2000). The frequency of neural oscillations that are evoked in response to olfactory stimuli can be affected by NO. The frequency of neural oscillations within the protocerebrum of the slug, *Limax maximus,* can be increased by injecting the substrate for NO synthesis, L-arginine (Gelperin *et al.* 1996). Conversely, decreasing the production of NO by injecting L-NAME into the protocerebrum inhibited the production of neural oscillations in response to olfactory stimuli (Gelperin *et al.* 1996). Studies in the honeybee, *Apis mellifera*, have also shown that blocking NO synthesis by injecting L-NAME into the protocerebrum impairs long-term memory of aversive olfactory stimuli (Müller, 1996), suggesting that NO has a role in olfactory processing, olfactory memory formation, and learning in invertebrates (Kendrick *et al.* 1997).

### 6.1b The role of NO in contact chemosensory-related behaviours

A growing number of studies have implicated NO in behavioural responses to contact chemosensory stimuli (Huque and Brand, 1994). In vertebrates, the presence of NOS has been confirmed in the taste organs of the channel catfish, *Ictaluras punctatus* (Huque and Brand, 1994) and the taste buds (vallate and foliate papilla) of rats (Kretz *et al.* 1998; Krizhanovsky *et al.* 2000).

In invertebrates, evidence of a role for NO in feeding is found in the central neuronal networks that underlie the feeding movements in molluscs (Straub *et al.* 2002). In the pond snail *Lymnaea stagnalis*, stimulating the mouthparts with a sucrose solution resulted in the generation of NO around the buccal ganglia when recorded via an NO meter (Kobayashi *et al.* 2000). As sucrose solutions are known to stimulate feeding in invertebrates (Dethier, 1976), this suggests that NO may act to initiate or maintain feeding.

It is also thought likely that NO is involved in learning and memory processes associated with feeding. In the sea hare, *Aplysia,* decreasing NO by injecting L-NAME into intact animals, abolished the formation of short, medium and long term memory to avoid an aversive feeding stimulus (Katzoff *et al.* 2002). Feeding on the aversive food was therefore maintained, despite being pre-conditioned to avoid it. Preventing NO synthesis by inhibiting NOS therefore altered the normal behavioural response to an aversive chemical stimulus.

Although NO has been shown to modulate behavioural responses to both odours and contact chemosensory stimuli, the subsequent signalling pathways influencing chemosensory input have not been identified. Recently, Murata *et al.* (2004) and Nakamura *et al.* (2005) predicted that the NO/cGMP signalling cascade was involved in chemosensory transduction of sucrose stimuli, although direct pharmacological evidence for the involvement of the NO/cGMP pathway is lacking.

As the oviposition digging rhythm has been previously shown to respond to contact chemosensory stimuli (see *Chapter 4*), the oviposition behaviour represents an ideal behaviour to analyse the role of NO on chemosensory input. This chapter therefore investigates the role of NO in the modulation of motor output of the digging rhythm, when the ovipositor valves are stimulated with 4 different

chemicals, NaCl, sucrose, HQ and LG. This chapter also investigates the possibility that NO acts via PKG to modulate behavioural responses to sucrose.

## 6.2 Materials and methods

### 6.2a Locusts

Female desert locusts, *Schistocerca gregaria* (Forskål) maintained and reared as described in *Chapter 2*.

### 6.2b Physiological methods

Recordings of the opener muscles and drug application to the terminal abdominal ganglion were performed as described in *Chapter 3* and *Chapter 5*.

### 6.2c Chemical stimulation

Four chemicals were repeatedly applied to the ventral surface of the ovipositor valves at 3 min intervals with a Pasteur Pipette. In an experiment a single concentration of each chemical was used to repeatedly stimulate the valves and the duration for which the rhythm stopped determined from muscle recordings. This analysis provided a baseline with which to compare experiments where the NO levels within the terminal abdominal ganglia were pharmacologically manipulated while repeatedly applying chemical stimuli to the valves. The chemicals used for stimulation of the ovipositor valves were 250mM NaCl, 1 M sucrose, 100mM HQ and 2 M LG. A total of 13 chemical stimulations were made to the ovipositor valves per experiment.

### 6.2d Drug application

Drugs were applied to the terminal abdominal ganglia (abdominal ganglion 7 and last abdominal ganglion), where the CPG network that underlies the digging rhythm is located (Thompson, 1986a, b). All drugs were applied after the 3$^{rd}$ presentation of a chemical stimulus and lasted for a duration of 10 min. Preliminary

experiments were performed to establish the duration taken for the optimal effects of the drugs to be expressed, and these were maximal after stimulus 8 (1440 s). These were compared to control (stimulus 3, 180 s) and wash, (stimulation 13, 2340 s). The effect of the NOS inhibitor, N-nitro-L-arginine methyl ester (L-NAME) and the nitric oxide donor (NOC-15) 3-(2-Hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPANONOate) on cessation of the digging rhythm were performed for all 4 chemicals (NaCl, sucrose, HQ and LG).

The pathways by which NO affects the behavioural responses to NaCl and sucrose were also investigated. For both chemicals, the effect of the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and the membrane permeable analogue of cGMP, 8-Bromoguanosine 3':5'-cyclic monophosphate (8-Br-cGMP) on the oviposition rhythm were investigated. An investigation of potential downstream molecular targets of cGMP was also carried out for both NaCl and sucrose responses. For this purpose, the generic protein kinase inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) was bath applied to the terminal abdominal ganglion. The PKG blocker, (9S, 10R, 12R)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9, 12-epoxy-1*H*-di indolo[1,2,3-fg:3', 2', 1',-kl]pyrrolo[3,4-I][1,6]benzodiazocine-10-carboxylic acid, methyl ester (KT-5823) was also used for sucrose stimulation experiments.

### 6.2e Statistical methods

The effect of drug application on the period for which the rhythm stopped after chemical stimulation (test periods compared to control periods) was tested using Student T-tests. Where data sets were non-parametric, Mann-Whitney U-Tests were used. To test the differences between the effects of control (without drug application to the terminal abdominal ganglion), L-NAME and PAPANONOate on the rhythm

for control, test and wash times, a Kruskal-Wallis test was appropriate because of the large variations in the data set. Where data sets were parametric, a one-way analysis of variance (ANOVA) was used. The association between preceding cycle frequency and the subsequent duration a rhythm stopped was tested using a Pearson Product Moment Correlation analysis.

## 6.3 Results

The effect of NO on the motor output representing the digging rhythm was analysed in response to chemical stimulation of the ovipositor valves with sucrose, LG, NaCl and HQ and, in addition, the signalling pathways through which NO mediates its effects analysed.

### 6.3a NO modulates the behavioural response to NaCl

The ovipositor valves were repeatedly stimulated with 250mM NaCl and the effect on the cessation of the digging rhythm analysed. The duration for which the digging rhythm stopped was not statistically different between control (23.28 ± 6.56s) and test (14.56 ± 4.1s) time periods (mean ± S.E.M, n = 10 animals; $p > 0.05$, Mann-Whitney U-test) (Fig. 6.1A). A recording from a ventral opener muscle showed that the duration between NaCl stimulation and the return of the digging rhythm steadily decreases between control (C), test (T) and wash (W) time periods. A recording of the ventral opener muscle showed that the response to control NaCl stimulation varied little over time (Fig 6.1B).

Bathing the terminal abdominal ganglion with 20mM L-NAME while repeatedly stimulating the ovipositor valves with 250mM NaCl, however, resulted in a significant increase in the duration for which the digging rhythm stopped, from a control value of 10.18 ± 3.09s to a test value of 32.59 ± 5.85s (mean ± S.E.M, n = 5 animals; Students T-test, t = -3.39, $p < 0.05$) (Fig. 6.1C). After 10 min of L-NAME bath application to the terminal abdominal ganglion, the duration for which the digging rhythm stopped increased significantly. After washing, the duration of cessation of the digging rhythm began to decrease and approach its control value (Fig. 6.1D).

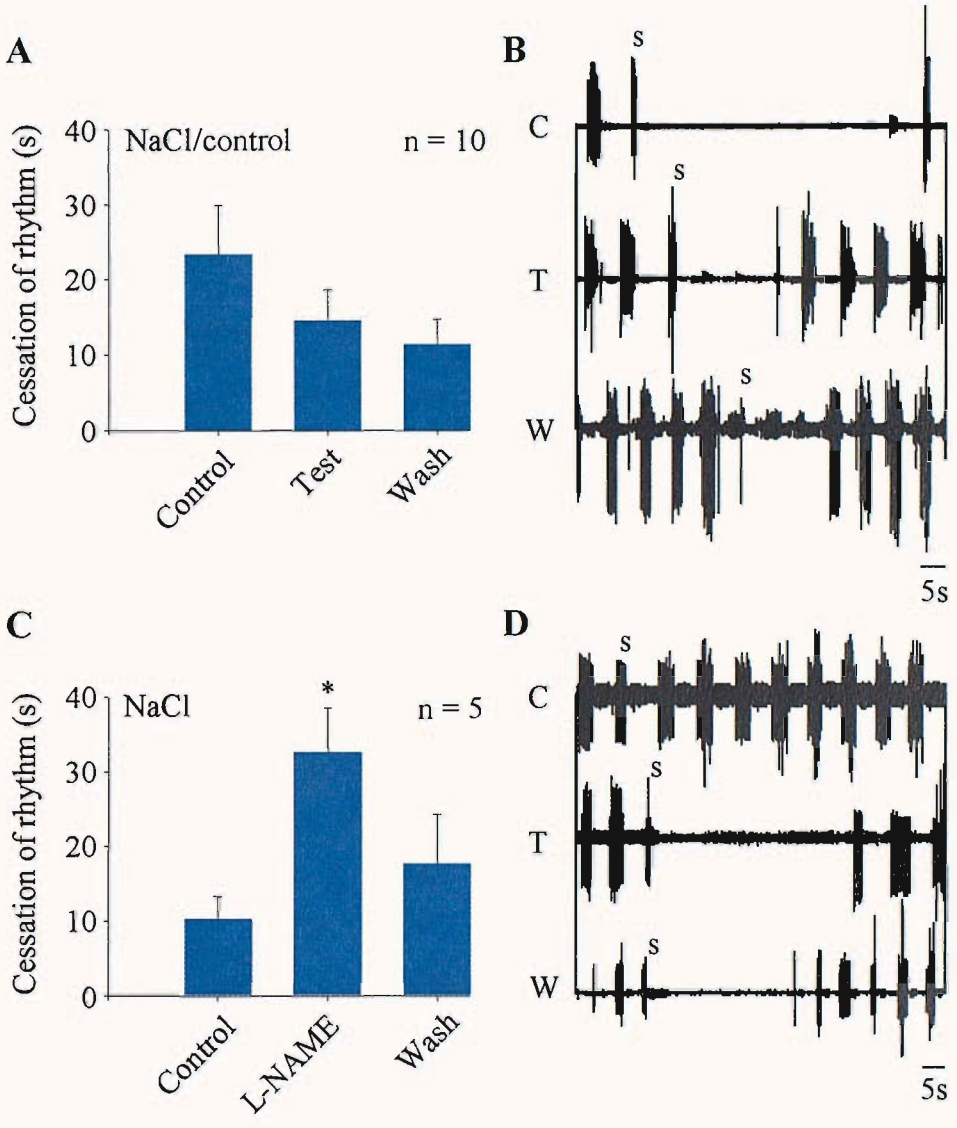



**Figure 6.1. NO modulates the response of the digging rhythm to NaCl. A.** Repetitive stimulation of the ovipositor valves with 250mM NaCl while simultaneously bathing the terminal abdominal ganglion in locust saline, showed no statistically significant difference in cessation of the digging rhythm, between control (23.28 ± 6.56) and test (14.56 ± 4.1) periods (mean ± S.E.M. $p > 0.05$, Mann-Whitney U-Test). **B.** A recording of opener muscle activity showing control (C), test (T) and wash (W) periods of chemical stimulation. The duration between chemical stimulation and the return of muscle activity gradually decreased on repeated chemical stimulation. **C.** Repetitive stimulation of the ovipositor valves with 250mM NaCl while simultaneously bathing the terminal abdominal ganglion with 20mM L-NAME, resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from a mean duration of 10.18 ± 3.09s to 32.59 ± 5.85s (mean ± S.E.M., Students T-test, t = -3.39, $p < 0.05$). **D.** After a 10 min application of L-NAME, opener muscle activity stopped for longer durations when chemically stimulated compared to control (* = $p < 0.05$).

Increasing the levels of NO by bath application of PAPANONOate to the terminal abdominal ganglion, resulted in a statistically significant decrease in the duration for which the rhythm stopped, from a control value of 17.96 ± 6.21s to a test value of 3.01 ± 0.83s (mean ± S.E.M, n = 6 animals; Students T-test, t = 2.38, $p < 0.05$) (Fig. 6.2A). A recording of ventral opener muscle activity showed that the digging rhythm stopped during the control period, when the ovipositor valves were stimulated with 250mM NaCl. Following a 10 min application of 0.2mM PAPANONOate, there was no cessation in the digging rhythm (Fig. 6.2B).

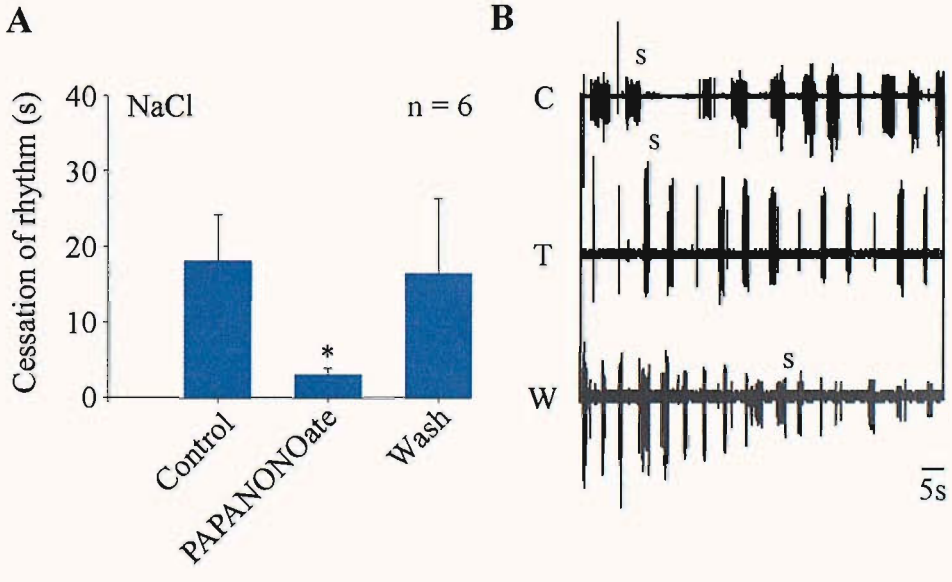



**Figure 6.2. Increasing NO levels within the terminal abdominal ganglion resulted in a decrease in the duration for which the digging rhythm stopped when the ovipositor valves were stimulated with 250mM NaCl. A.** Bathing the terminal abdominal ganglion with the NO donor PAPANONOate while repeatedly stimulating the valves with 250mM NaCl significantly decreased the duration for which the digging rhythm stopped, from a control of 17.96 ± 6.21s to 3.01 ± 0.83s (mean ± S.E.M., Students T-test, t = 2.38, $p < 0.05$). **B.** Stimulating the digging rhythm with 250mM NaCl when the terminal abdominal ganglion has been bathed for 10 min with 0.2mM PAPANONOate (T), showed a significant decrease in the duration of the cessation of the digging rhythm compared to control stimulation. When chemically stimulated, rhythmic opener muscle activity was therefore sustained and continued when NO levels within the terminal abdominal ganglion were increased. When PAPANONOate was washed from the terminal abdominal ganglion of this particular animal, the effects of NO were not reversible.

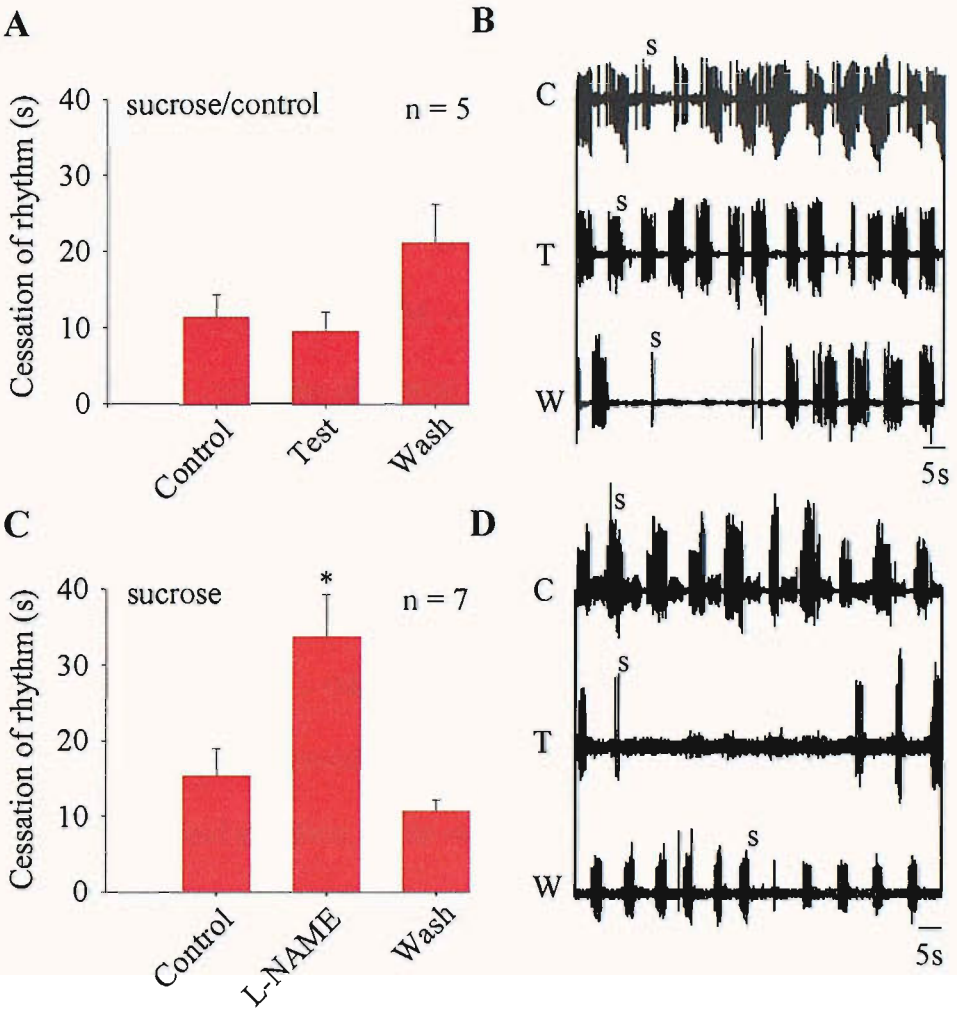



**Figure 6.3. NO modulates the response of the digging rhythm to sucrose stimulation. A.** The ovipositor valves were repeatedly stimulated with 1 M sucrose while bathing the terminal abdominal ganglion with locust saline. No statistically significant difference between control (11.37 ± 2.93s) and test (9.45 ± 2.55s) values were observed for the cessation of the digging rhythm (mean ± S.E.M., Students T-test; $t = 0.5$, $p > 0.05$). **B.** A recording of opener muscle activity showed no difference between control and test values for cessation of the digging rhythm. The wash period (20.87 ± 5.23s) however, showed an increase in the cessation of the digging rhythm compared to the control and test period, although this difference was not statistically significant (Students T-test; $t = -2.03$, $p > 0.05$). **C.** Repetitive stimulation of the ovipositor valves with 1 M sucrose while simultaneously bathing the terminal abdominal ganglion with 20mM L-NAME, resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from 15.16 ± 3.76s to 33.56 ± 5.69s (mean ± S.E.M., Students T-test, $t = -2.77$, $p < 0.05$). **D.** A 10 min application of L-NAME to the terminal abdominal ganglion resulted in an increase in the cessation of opener muscle activity when chemically stimulated compared to control.

### 6.3b NO modulates the behavioural response to sucrose

The ovipositor valves were repeatedly stimulated with 1 M sucrose at 3 min intervals and the effect on the cessation of the digging rhythm analysed. There were no statistically significant differences between control (11.37 ± 2.93s) and test durations (9.45 ± 2.55) (mean ± S.E.M, n = 5 animals; Students T-test, t = 0.5, $p > 0.05$) (Fig. 6.3A). A recording of ventral opener muscle activity showed no statistically significant difference in the cessation of the rhythm, between control and test values. The wash period however, showed a distinct increase in the duration for which the rhythm stopped, although the difference was not statistically significant (Students T-test, t = -2.03, $p > 0.05$) (Fig. 6.3B).

NO levels were decreased by bath applying the NOS inhibitor L-NAME to the terminal abdominal ganglion, while repeatedly stimulating the ovipositor valves with 1 M sucrose. This resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from 15.16 ± 3.76s to 33.56 ± 5.69s (mean ± S.E.M, n = 7 animals; Students T-test, t = -2.77, $p < 0.05$) (Fig. 6.3C). An example of a recording of ventral opener muscle activity showed that 20mM L-NAME increased the cessation of the rhythm (Fig. 6.3D).

Increasing NO levels by bath application of 0.2mM PAPANONOate to the terminal abdominal ganglion resulted in a statistically significant decrease in the duration for which the digging rhythm stopped when the ovipositor valves were repeatedly stimulated with 1 M sucrose. The duration of cessation of the digging rhythm decreased from a control of 16.08 ± 4.47s to 2.81 ± 0.67s (mean ± S.E.M, n = 8 animals; $p < 0.05$, Mann-Whitney U-test) (Fig. 6.4A). An example of a recording from the ventral opener muscle showed that sucrose stimulation during PAPANONOate application had no effect on the rhythm (Fig 6.4B).

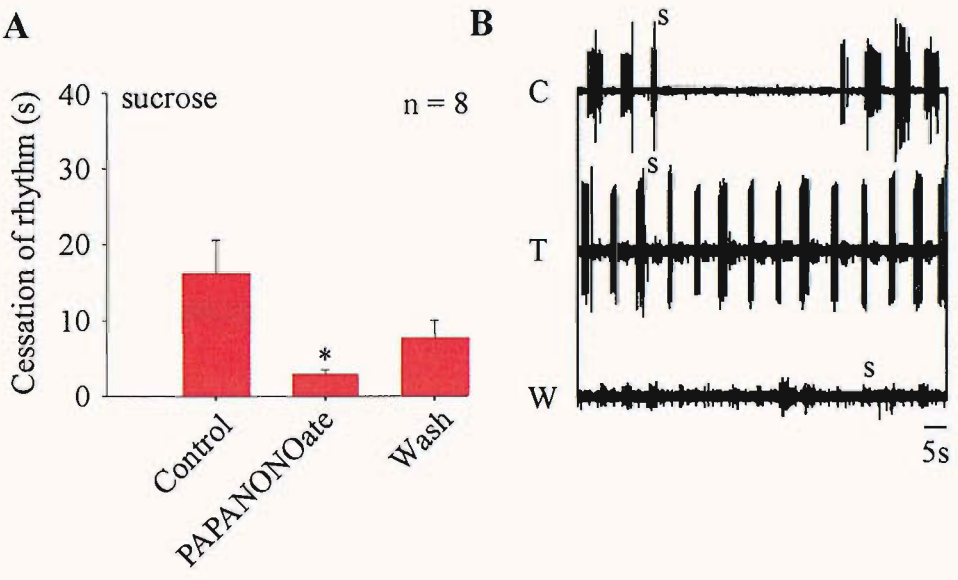



**Figure 6.4. Increasing NO levels within the terminal abdominal ganglion resulted in a decrease in the duration for which the digging rhythm stopped when the ovipositor valves were stimulated with 1 M sucrose. A.** Bathing the terminal abdominal ganglion with PAPANONOate while repeatedly stimulating the ovipositor valves with 1 M sucrose significantly decreased the cessation of the digging rhythm from 16.08 ± 4.47s to 2.81 ± 0.67s (mean ± S.E.M., $p < 0.01$, Mann-Whitney U-Test). **B.** A recording of opener muscle activity showed a significant decrease in the duration of cessation of the digging rhythm when the ovipositor valves were stimulated with 1 M sucrose while the terminal abdominal ganglion had been bathed with 0.2mM PAPANONOate (T) compared to control (C) and wash (W).

### 6.3c NO modulates the behavioural response to HQ

Repetitive stimulation of the ovipositor valves with 100mM HQ, resulted in no statistical difference between control (6.27 ± 2.01s) and test (6.41 ± 2.5s) time periods (mean ± S.E.M, n = 5 animals; $p > 0.05$, Mann-Whitney U-test) (Fig. 6.5A). Similarly a recording of ventral opener muscle activity showed little difference in the cessation of the digging rhythm between control and test periods (Fig. 6.5B). Decreasing NO levels by bath applying 20mM L-NAME, resulted in a statistically significant increase in the duration for which the rhythm stopped, from a control value of 6.45 ± 2.43s to a test value of 24.79 ± 6.07s (mean ± S.E.M, n = 5 animals; $p < 0.05$, Mann-Whitney U-test) (Fig. 6.5C). A recording of ventral opener muscle activity showed an increase in the duration of the cessation of the digging rhythm

after 100mM HQ stimulation while the terminal abdominal ganglion was bath applied with 20mM L-NAME, compared to control stimulation (Fig. 6.5D).

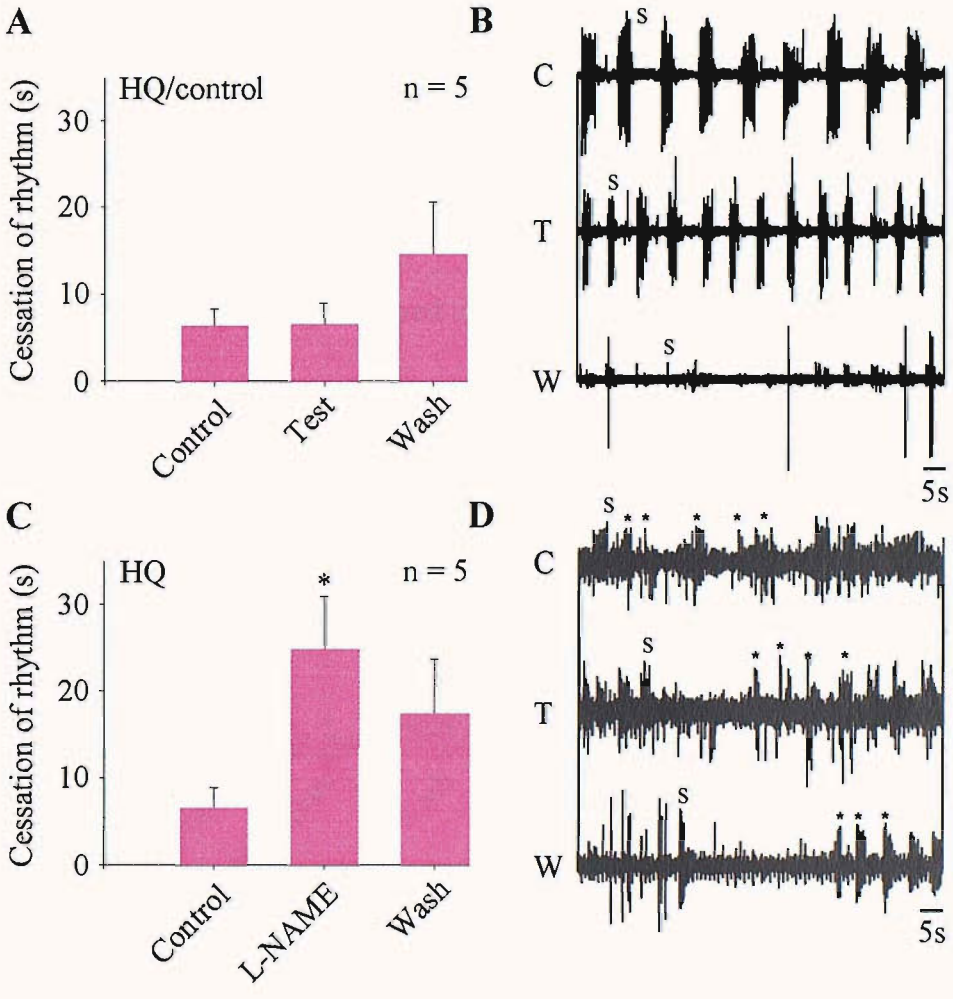



**Figure 6.5. NO modulates the effects of HQ on the digging rhythm. A.** Repetitive stimulation of the ovipositor valves with 100mM HQ while bathing the terminal abdominal ganglion with locust saline had no effect on the cessation of the rhythm over time (control, 6.27 ± 2.01s, test, 6.41 ± 2.5s) (mean ± S.E.M., $p > 0.05$, Mann-Whitney U-Test). **B.** A recording of opener muscle activity showed no difference in the duration of cessation of the digging rhythm between control (C) and test (T) time periods. The wash period (W) (14.44 ± 6.12s) showed an increase in cessation of the digging rhythm, although this difference was not statistically different from the control and test time periods ($p > 0.05$, Mann-Whitney U-Test). **C.** Repetitive stimulation of the ovipositor valves while simultaneously bathing the terminal abdominal ganglion with 20mM L-NAME resulted in a statistically significant increase in the duration of cessation of the digging rhythm from a mean of 6.45 ± 2.43s to 24.79 ± 6.07s (mean ± S.E.M., $p < 0.05$, Mann-Whitney U-test). **D.** Bathing the terminal abdominal ganglion for 10 min with 20mM L-NAME resulted in the rhythm stopping for longer durations than control. Due to the poor nature of the recording, the return of rhythmic activity matching the rhythm preceding chemical stimulation is marked with a (*). Washing L-NAME from the terminal abdominal ganglion did not result in a complete return to control in this example.

Conversely increasing NO levels within the terminal abdominal ganglion by bath applying 0.2mM PAPANONOate, while repeatedly stimulating the ovipositor valves with HQ resulted in a significant decrease in the duration for which the digging rhythm stopped, from a control value of 7.63 ± 2.62s to 2.23 ± 0.37s (mean ± S.E.M, n = 10 animals; $p < 0.05$, Mann-Whitney U-test) (Fig. 6.6A). A recording of ventral opener muscle activity showed a decrease in the cessation of opener muscle activity after 100mM HQ stimulation, when the terminal abdominal ganglion was bathed with PAPANONOate, compared to control stimulation (Fig. 6.6B).

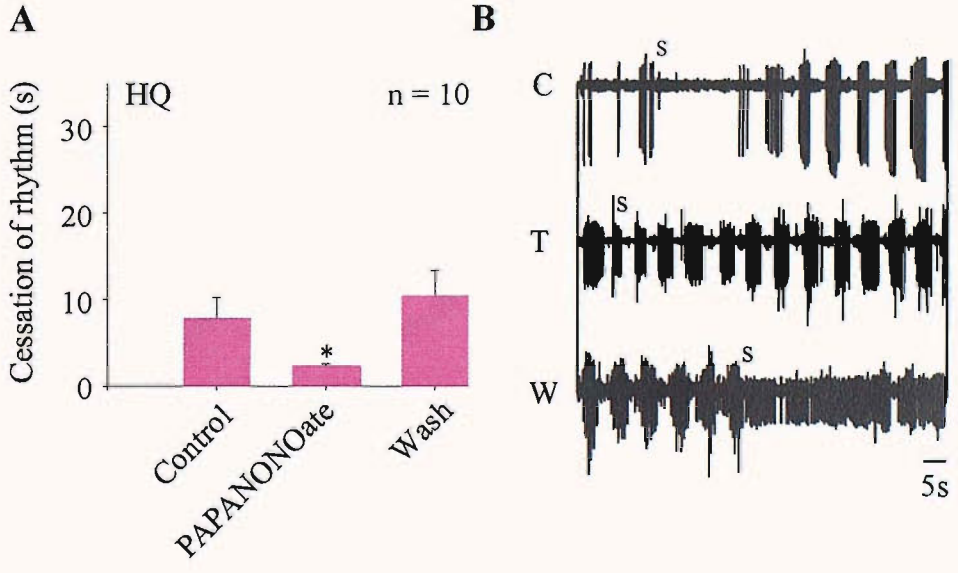



**Figure 6.6. Increasing NO levels within the terminal abdominal ganglion resulted in a decrease in the duration of cessation of the digging rhythm when the ovipositor valves were stimulated with 100mM HQ. A.** Bathing the terminal abdominal ganglion for 10 min with 0.2mM PAPANONOate while repeatedly stimulating the ovipositor valves with 100mM HQ decreased the duration of cessation of the digging rhythm, from 7.63 ± 2.62s to 2.23 ± 0.37s (mean ± S.E.M., $p < 0.05$, Mann-Whitney U-Test). **B.** A recording of muscle activity showing a decrease in the duration of cessation of the digging rhythm when the terminal abdominal ganglion was bathed with 0.2mM PAPANONOate (C, control, T, test, W, wash).

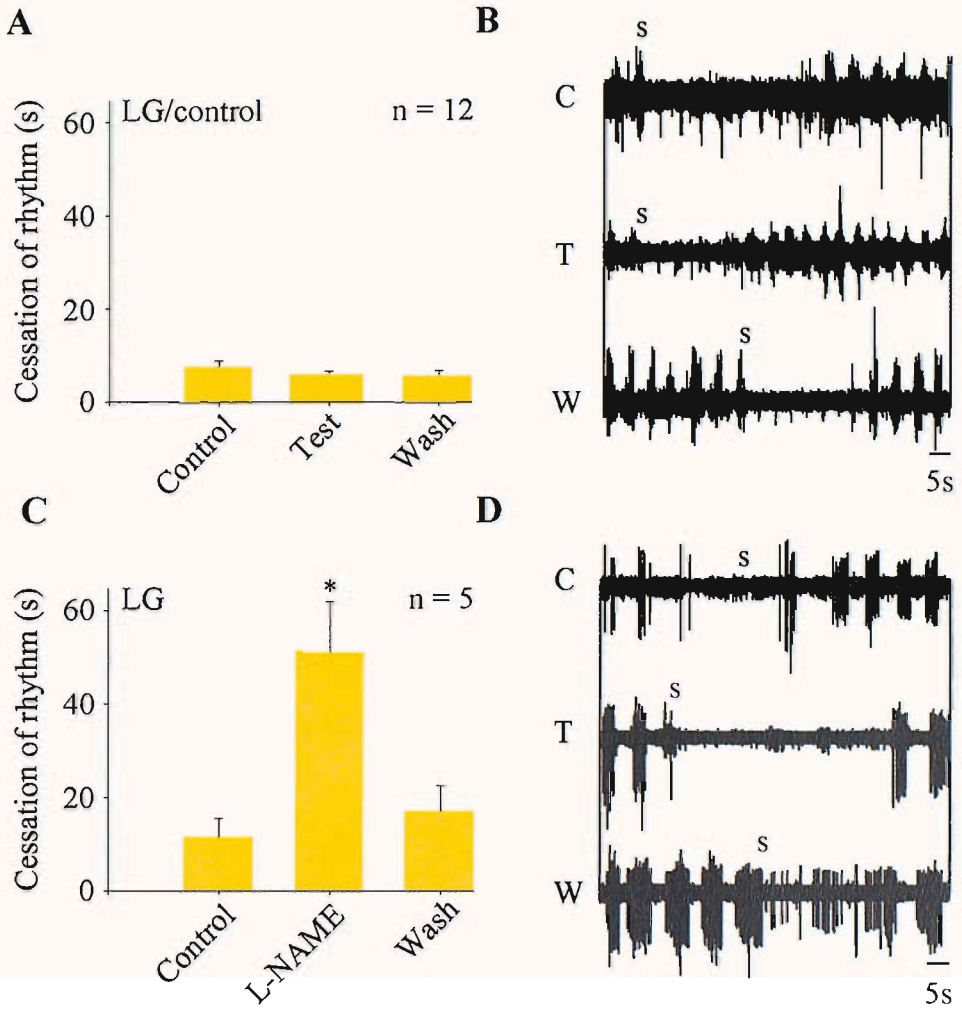



**Figure 6.7. The effect of decreasing NO availability in the terminal abdominal ganglion, on the responses of the digging rhythm when the ovipositor valves are stimulated with LG. A.** Repetitive stimulation of the ovipositor valves with 2 M LG while bathing the terminal abdominal ganglion with locust saline showed no statistically significant difference between control (7.32 ± 1.58s) and test values (5.79 ± 0.8s, mean ± S.E.M) for cessation of the digging rhythm (Students T-test; t = 0.86, $p > 0.05$). **B.** A recording of opener muscle activity showed no difference in cessation of the digging rhythm between control (C), test (T) and wash (W) time periods of LG stimulation. **C.** Bathing the terminal abdominal ganglion for 10 min with the NOS inhibitor L-NAME, resulted in a statistically significant increase in the duration of cessation of the digging rhythm, from a control value of 11.03 ± 4.49s to 51.04 ± 10.86s (mean ± S.E.M, Students T-test, t = -3.4, $p < 0.01$). **D.** A recording of opener muscle activity showing an increase in cessation of the digging rhythm when the ovipositor valves were stimulated with LG, while the terminal abdominal ganglion was bathed with 20mM L-NAME.

### 6.3d The effect of NO on the behavioural responses to LG

Repeated stimulation of the ovipositor valves with 2 M LG stopped the rhythm, the duration of which did not change over time (Control, 7.32 ± 1.58s; test, 5.79 ± 0.8s) (mean ± S.E.M, n = 12 animals; Students T-test, t = 0.86, $p > 0.05$) (Fig.

6.7A). A recording of the ventral opener muscle showed no observable difference between control, test and wash values (Fig. 6.7B). Decreasing NO levels in the terminal abdominal ganglion by bath applying 20mM L-NAME resulted in a statistically significant increase in the duration for which the digging rhythm stopped, from 11.03 ± 4.49s to 51.04 ± 10.86s (mean ± S.E.M, n = 5 animals; Students T-test, t = -3.4, $p < 0.05$) when the ovipositor valves were stimulated with 2 M LG (Fig. 6.7C). A recording of ventral opener muscle activity showed an increase in the duration of cessation of the digging rhythm, compared to that of control and wash time periods (Fig. 6.7D).

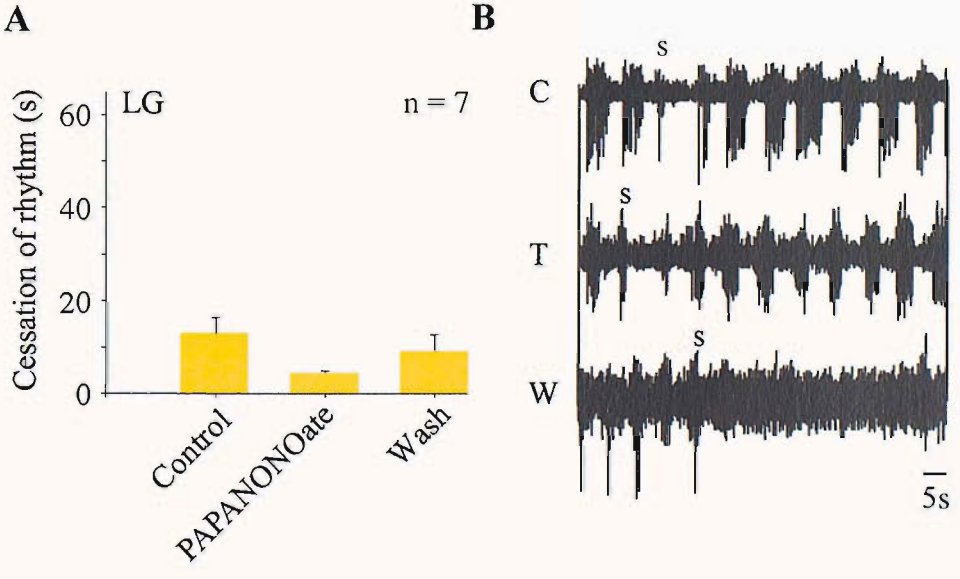



**Figure 6.8. The effect of increased NO levels on the digging rhythms response to LG stimulation of the ovipositor valves. A.** Bathing the terminal abdominal ganglion with PAPANONOate while repeatedly stimulating the ovipositor valves with 2 M LG resulted in a decrease in cessation of the digging rhythm (control, 12.56 ± 3.71s; test, 3.99 ± 0.89s, mean ± S.E.M., $p > 0.05$, Mann-Whitney U-Test). **B.** A recording of opener muscle activity when the terminal abdominal ganglion was bathed for 10 min with 0.2mM PAPANONOate. The recording shows a decrease in cessation of the digging rhythm, between control (C) and test (T) time periods, when the terminal abdominal ganglion was bathed with 0.2mM PAPANONOate and the ovipositor valves were chemically stimulated with 2 M LG (wash, W).

Increasing NO levels by bath applying 0.2mM PAPANONOate to the terminal abdominal ganglion, while repeatedly stimulating the ovipositor valves with

2 M LG however, showed a small reduction in the duration of cessation of the rhythm from

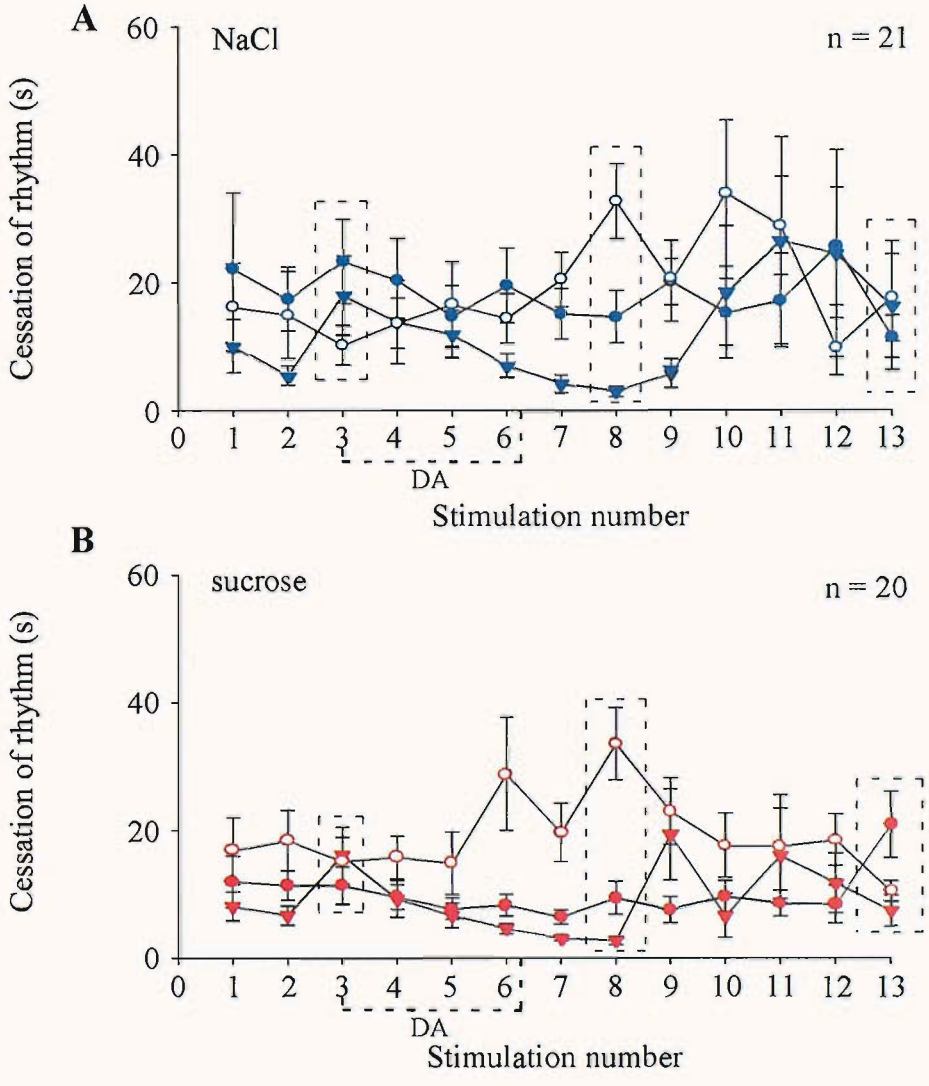



**Figure 6.9. Summary of the effects of NO on repetitive chemical stimulation of the ovipositor valves. Periods of drug application are denoted by "DA." A.** Repetitive stimulation of the ovipositor valves with 250mM NaCl while NO levels in the terminal abdominal ganglion were increased or decreased showed significant effects on the cessation of the digging rhythm. Control, test and wash periods are stimulation numbers 3, 8 and 13 respectively. Test periods showed statistically significant differences between cessation of the digging rhythm between all 3 sets of experiments (Kruskal-Wallis, $K = 10.33$, $p < 0.01$). No statistically significant differences in cessation of the digging rhythm were observed between control ($K = 1.15$, $p > 0.05$) and wash ($K = 1.59$, $p > 0.05$) periods for control (closed circles), L-NAME (open circles) and PAPANONOate experiments (triangles). **B.** Repetitive stimulation of the ovipositor valves with 1 M sucrose while increasing or decreasing NO levels within the terminal abdominal ganglion had significant effects on the cessation of the digging rhythm. Test time periods showed statistically significant

differences in cessation of the digging rhythm between the 3 sets of data ($K = 13.23$, $p < 0.01$). No statistically significant differences in cessation of the digging rhythm were observed between control ($K = 0.8$, $p > 0.05$) and wash (One-way ANOVA, $F_{2, 17} = 2.38$, $p > 0.05$) periods of chemical stimulation, for control (closed circles), L-NAME (open circles) and PAPANONOate (triangles) experiments ($p > 0.05$).

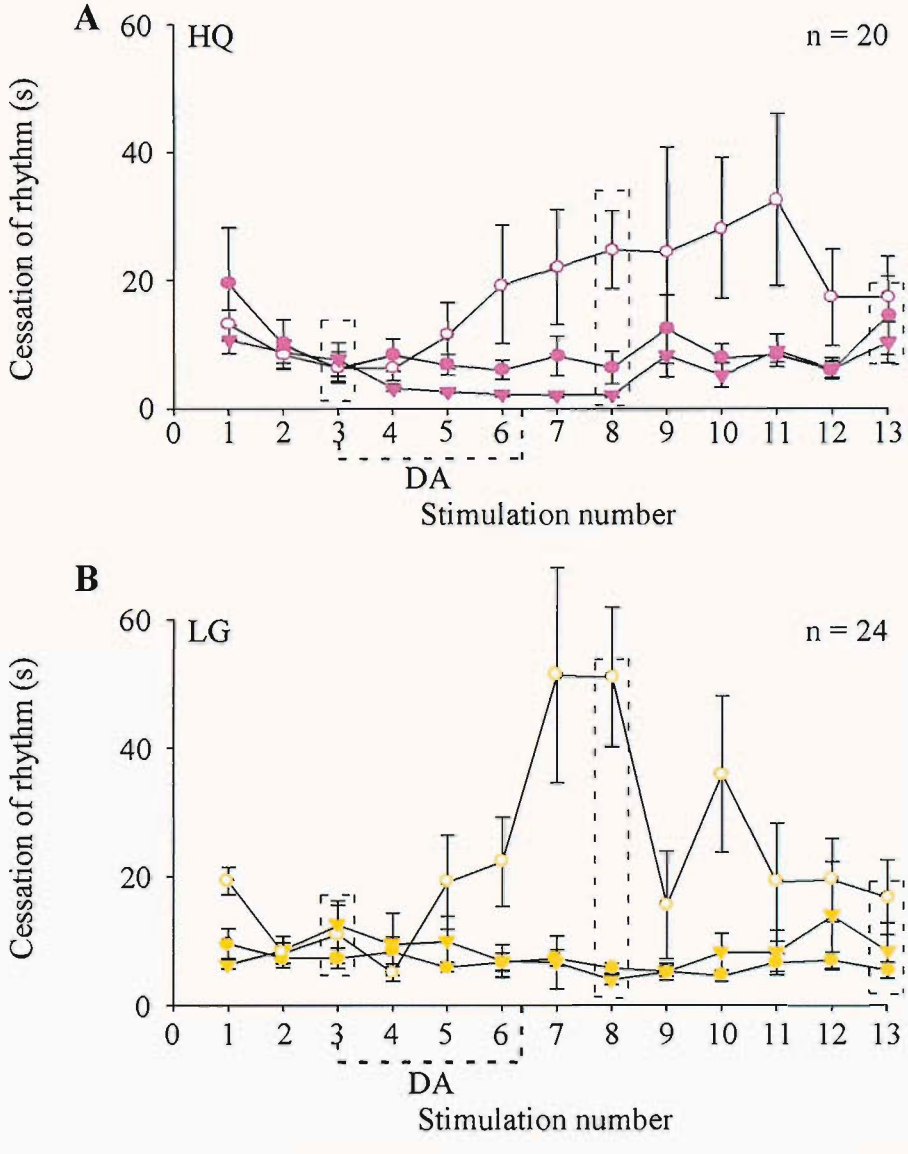



**Figure 6.10. Summary showing the effect of NO on repetitive stimulation of the ovipositor valves. A.** Repetitive stimulation of the ovipositor valves with 100mM HQ while NO levels in the terminal abdominal ganglion were increased or decreased, showed significant effects on the cessation of the digging rhythm. Test periods showed statistically significant differences in cessation of the digging rhythm, between all 3 sets of experiments ($K = 13.59$, $p < 0.01$). No statistically significant differences in cessation of the digging rhythm were observed between control ($K = 0.25$, $p > 0.05$) and wash (One-way ANOVA, $F_{2, 18} = 0.6$, $p > 0.05$) periods for control (closed circles), L-NAME (open circles) and PAPANONOate (triangles) experiments. **B.** Repetitive stimulation of the ovipositor valves with 2 M LG while increasing or decreasing NO levels, showed significant effects on the digging rhythm. Test and wash periods showed statistically significant differences in

cessation of the digging rhythm between all 3 sets of experiments for control (closed circles), 20mM L-NAME (open circles) and PAPANONOate (triangles) time periods (test, Kruskal-Wallis, $K = 12.32$, $p < 0.05$; wash, $K = 11.09$, $p < 0.05$). No statistically significant differences were observed between control time periods for all 3 data sets ($K = 0.66$, $p > 0.05$).

12.56 ± 3.71s to 3.99 ± 0.89s (mean ± S.E.M, n = 7 animals, $p < 0.05$, Mann Whitney U-test) (Fig. 6.8A, B).

Figures 6.9 and 6.10 show a summary of all the effects of L-NAME and PAPANONOate application on the cessation of the oviposition digging rhythm caused by all 4 test chemicals. Both graphs show the time course of chemical stimulation and the periods of drug application. After 10 min drug application, all 4 chemicals showed statistically significant differences between control, L-NAME and PAPANONOate treated animals (NaCl, $K = 10.3$, $p < 0.05$; sucrose, $K = 13.2$, $p < 0.05$; HQ, $K = 13.6$, $p < 0.05$ and LG, $K = 12.3$, $p < 0.05$). LG, $K = 12.3$, $p < 0.05$).

No statistically significant differences were found for control time periods for all 4 chemicals, between control, L-NAME treated and PAPANONOate treated animals (NaCl, $K = 1.2$, $p > 0.05$; sucrose, $K = 0.8$, $p > 0.05$; HQ, $K = 0.3$, $p > 0.05$ and LG, $K = 0.7$, $p > 0.05$). Similarly wash periods for 3 out of the 4 chemicals showed no statistically significant differences between control, L-NAME and PAPANONOate treated animals (NaCl, $K = 1.6$, $p > 0.05$; sucrose, One-way ANOVA, $F_{2, 17} = 2.4$, $p > 0.05$; HQ, $F_{2, 18} = 0.6$, $p > 0.05$). Despite washing all drug applications from the terminal abdominal ganglion, statistically significant differences were observed between control, L-NAME and PAPANONOate treated animals for LG ($K = 7.63$, $p < 0.05$), presumably due to the long time periods to wash the drugs from the central nervous system.

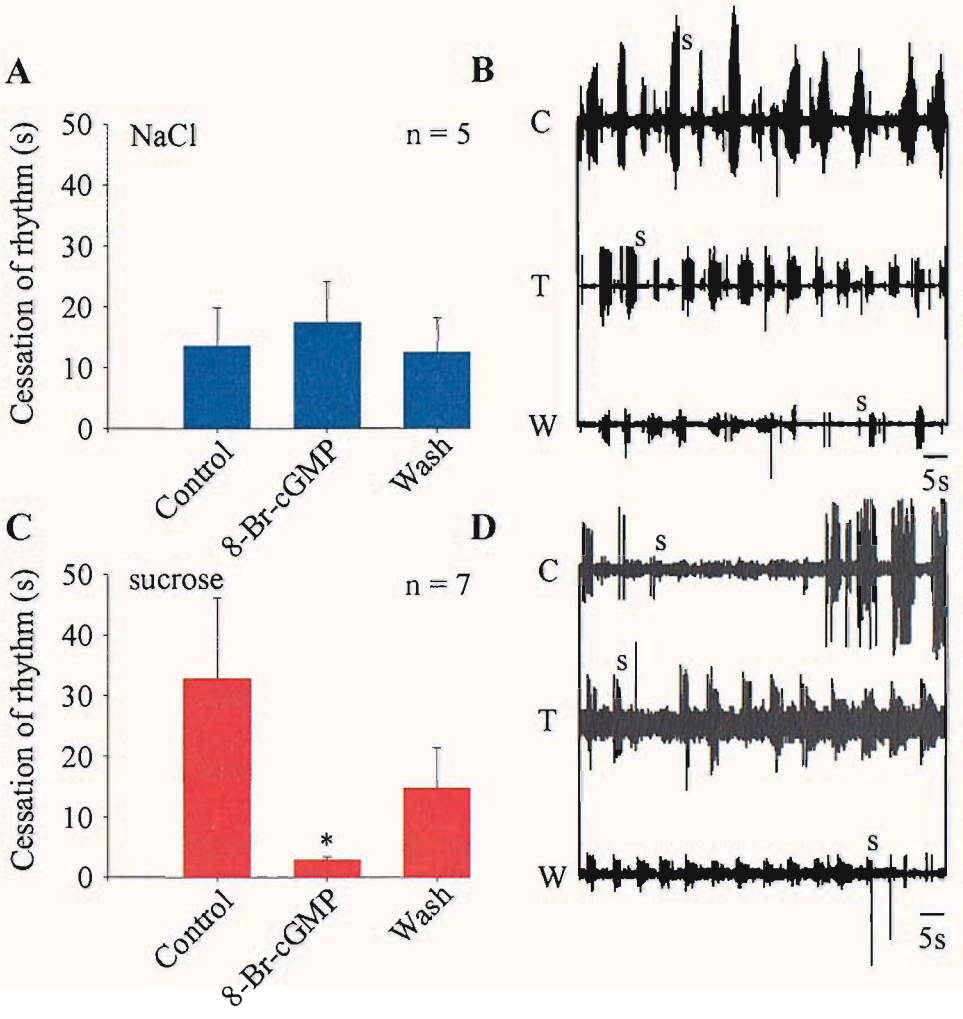



**Figure 6.11. The effect of the cGMP analogue, 8-Br-cGMP, on the motor output of the digging rhythm. A.** Bath application of 0.1mM 8-Br-cGMP while repeatedly stimulating the ovipositor valves with 250mM NaCl resulted in no statistically significant change in the cessation of the digging rhythm over time, between control (C) (13.4 ± 6.4s) and test (T) (17.37 ± 6.62s, mean ± S.E.M., Students T-test, t = -0.43, $p > 0.05$) time periods. **B.** A recording of opener muscle activity showed no difference in the cessation of the digging rhythm when the ovipositor valves were stimulated with NaCl. **C.** Bath application of 0.1mM 8-Br-cGMP while repeatedly stimulating the ovipositor valves with 1 M sucrose resulted in a statistically significant decrease in the cessation of the digging rhythm, from 32.55 ± 13.46s to 2.73 ± 0.6s (mean ± S.E.M., $p < 0.05$, Mann-Whitney U-Test). **D.** A recording of opener muscle activity showed a decrease in the cessation of the digging rhythm when the ovipositor valves were stimulated with sucrose in the presence of 8-Br-cGMP (W, wash).

### 6.3e Are the effects of NO on behavioural responses to NaCl and sucrose mediated through cGMP-dependent signalling pathways?

The application of the membrane permeable analogue of cGMP, 0.1mM 8-Br-cGMP, to the terminal abdominal ganglion, while repeatedly stimulating the

ovipositor valves with 250mM NaCl had no statistically significant effect on the duration of the cessation of the digging rhythm (control, 13.4 ± 6.4s; test, 17.37 ± 6.62s) (mean ± S.E.M, n = 5 animals; Students T-test, t = -0.43, $p > 0.05$) (Fig. 6.11A).

An example of a recording of the ventral opener muscle shows no difference in the cessation of the digging rhythm between control and test values (Fig. 6.11B). By contrast, bath application of 0.1mM 8-Br-cGMP to the terminal abdominal ganglion while repeatedly stimulating the valves with 1 M sucrose resulted in a statistically significant decrease in the duration of the cessation of the digging rhythm compared to control (mean ± S.E.M, n = 7 animals, control, 32.55 ± 13.46s; test, 2.73 ± 0.6s; $p < 0.05$, Mann-Whitney U-test) (Fig. 6.11C). Similarly, a recording of opener muscle activity showed a decrease in the duration of cessation of the digging rhythm during chemical stimulation with 1 M sucrose (Fig. 6.11D).

Activation of sGC by NO is known to result in a sustained increase in intracellular levels of cGMP (Bredt and Snyder, 1989). An inhibitor of sGC, 0.1mM ODQ, was bath applied to the terminal abdominal ganglion while the ovipositor valves were repeatedly stimulated with either NaCl or sucrose. A small increase in the cessation of the digging rhythm was observed for NaCl after a 10 min bath application of 0.1mM ODQ, from a control of 17.89 ± 3.3s to 24.93 ± 9.65s, although this increase was not statistically significant (mean ± S.E.M, n = 5 animals; $p > 0.05$, Mann-Whitney U-test) (Fig. 6.12A, B). Conversely, bath application of the terminal abdominal ganglion with 0.1mM ODQ resulted in a statistically significant increase in the cessation of the digging rhythm (control, 14.14 ± 4.15s; test, 60.79 ± 9.27s (mean ± S.E.M, n = 5 animals; Students T-test, t = -4.59, $p < 0.05$), when the ovipositor valves were repeatedly stimulated with sucrose (Fig. 6.12C). A recording of ventral opener muscle activity showed that when the terminal abdominal ganglion

is bathed with 0.1mM ODQ the cessation of the digging rhythm significantly increased when stimulated with 1 M sucrose compared to control stimulations (Fig. 6.12D).

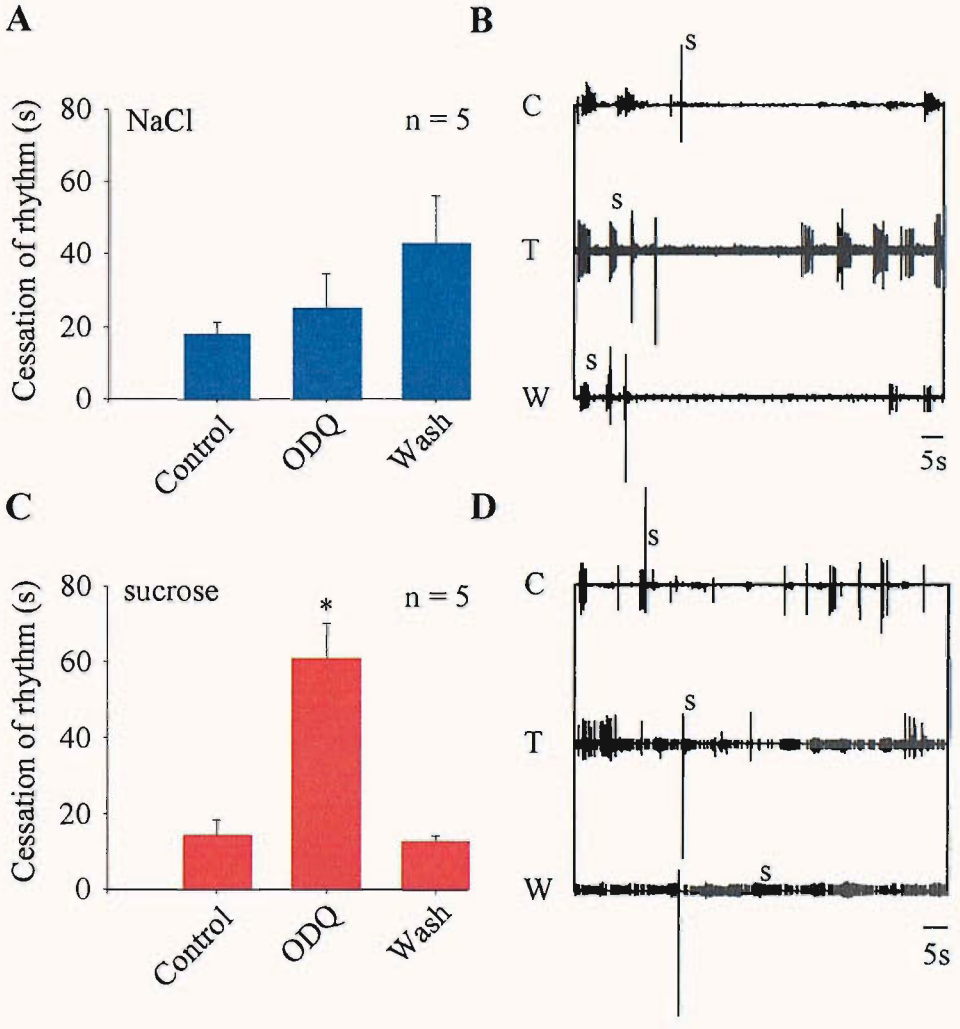



**Figure 6.12. The effect of blocking sGC activity on the digging rhythm. A.** Bath application of the sGC blocker, 0.1mM ODQ while repeatedly stimulating the ovipositor valves with 250mM NaCl, resulted in no statistically significant difference in cessation of the digging rhythm over time (control, 17.89 ± 3.3s, test, 24.93 ± 9.65s; mean ± S.E.M., $p > 0.05$, Mann-Whitney U-Test). **B.** A recording of opener muscle activity showed no difference in cessation of the digging rhythm, between control and test time periods after bath application of the terminal abdominal ganglion with 0.1mM ODQ while the ovipositor valves were repeatedly stimulated. **C.** Repetitive stimulation of the ovipositor valves with 1 M sucrose while bath applying the terminal abdominal ganglion with 0.1mM ODQ resulted in a statistically significant increase in the cessation of the digging rhythm, from a control value of 14.14 ± 4.15s to a test value of 60.79 ± 9.27s (mean ± S.E.M., Students T-test, t = -4.59, $p < 0.01$). **D.** Repetitive stimulation of the ovipositor valves while bath applying the terminal abdominal ganglion with ODQ (T) showed a clear increase in the cessation of muscle activity, compared to control (C) time periods (W, wash).

These results suggest that the effects of NO on the modulatory pathways of NaCl and sucrose input are mediated by different molecular mechanisms since the effects of NO on NaCl were cGMP-independent, whereas the effects on sucrose were cGMP-dependent.

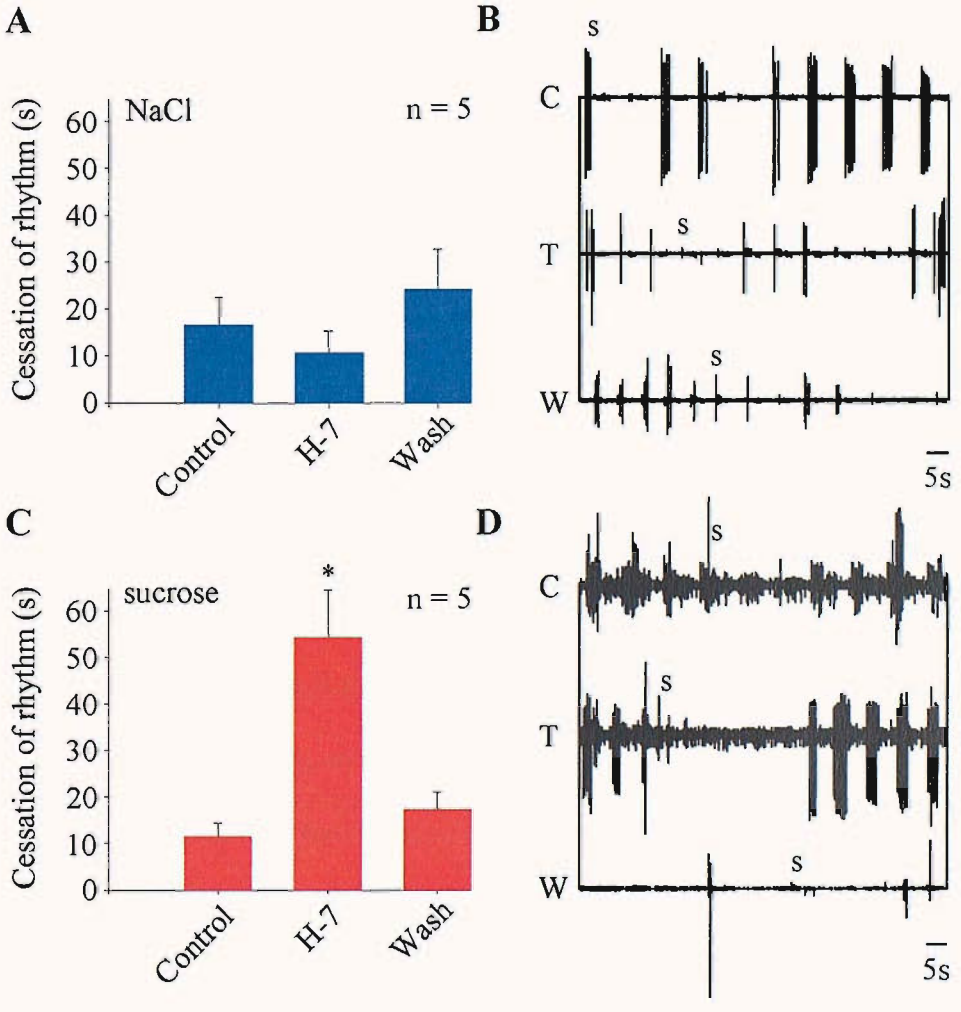



**Figure 6.13. The effect of the generic protein kinase blocker, H-7 on the response of the digging rhythm to NaCl and sucrose stimulation. A.** A 10 min bath application of the terminal abdominal ganglion with 0.1mM H-7, while repeatedly stimulating the ovipositor valves with 250mM NaCl, had no statistically significant effect on the cessation of the digging rhythm (control, 16.52 ± 5.91s; test, 10.55 ± 4.71s; mean ± S.E.M., $p > 0.05$, Mann-Whitney U-Test). **B.** A recording of opener muscle activity showed that repetitive stimulation of the ovipositor valves while bath applying 0.1mM H-7, had no effect on the cessation of the digging rhythm. **C.** A 10 min bath application of 0.1mM H-7 resulted in a statistically significant increase in the duration of cessation of the digging rhythm, from a control (C) value of 11.46 ± 2.89s to a test (T) value of 54.05 ± 10.43s (mean ± S.E.M., Students T-test, t = -3.93, $p < 0.01$) during sucrose stimulation. **D.** Repeatedly stimulating the ovipositor valves with 1 M sucrose while bath applying the terminal abdominal ganglion with 0.1mM H-7, caused a clear increase in the duration of cessation of the digging rhythm (W, wash).

To determine whether the NO effects on NaCl input could be dependent on protein kinases, the generic protein kinase inhibitor H-7 was bath applied. A 10 min bath application of 0.1mM H-7 resulted in no significant differences in cessation of the digging rhythm, between control (16.52 ± 5.91s) and test time periods (10.55 ± 4.71s; mean ± S.E.M, n = 5 animals; $p > 0.05$, Mann-Whitney U-test) for repetitive NaCl stimulation (Fig. 6.13A). Similarly, a recording from the ventral opener muscle showed no difference in the duration of cessation of the digging rhythm between control and test stimulations with 250mM NaCl (Fig. 6.13B).

The generic protein kinase inhibitor, H-7 was bath applied to the terminal abdominal ganglion while the ovipositor valves were repeatedly stimulated with 1 M sucrose. Bath application of 0.1mM H-7 to the terminal abdominal ganglion resulted in a statistically significant increase in the duration of cessation of the digging rhythm to sucrose stimulation, between control (11.46 ± 2.89s) and test (54.05 ± 10.43s) time periods (mean ± S.E.M, n = 5 animals; Students T-test = -3.93, $p < 0.05$) (Fig. 6.13C). Similarly, an example of a recording from the ventral opener muscle shows an increase in the duration of cessation of the rhythm after 10 min H-7 bath application compared to control stimulations (Fig. 6.13D).

### 6.3f Are behavioural responses to sucrose stimulation modulated by PKG?

To establish if the responses of the digging rhythm to sucrose stimulation are modulated via a NO/cGMP-PKG signalling pathway, the PKG inhibitor KT-5823 was bath applied to the terminal abdominal ganglion and the valves repeatedly stimulated with 1 M sucrose. A 10 min bath application with 10μM KT-5823 resulted in a statistically significant increase in the duration of cessation of the digging rhythm, from a control value of 15.08 ± 6.21s, to a test value of 66.1 ± 10.47s (mean ± S.E.M, n = 5 animals; $p < 0.05$, Mann-Whitney U-test) (Fig. 6.14A).

A recording of the ventral opener muscle showed an increase in the cessation of the digging rhythm for test stimulations of the ovipositor valves with 1 M sucrose compared to control (Fig. 6.14B).

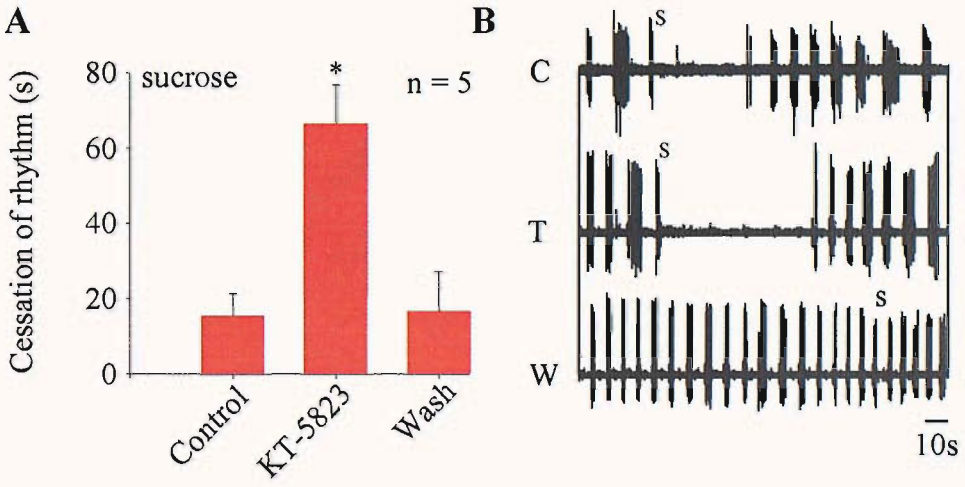



**Figure 6.14. The effect of the PKG blocker, KT-5823 on the response of the digging rhythm to sucrose stimulation of the ovipositor valves. A.** Bathing the terminal abdominal ganglion with 10μM KT-5823 while chemically stimulating the ovipositor valves with 1 M sucrose, significantly increased the cessation of the digging rhythm, from a control of 15.08 ± 6.21s to 66.1 ± 10.47s (mean ± S.E.M., $p < 0.05$ Mann-Whitney U-Test). **B.** A recording of opener muscle activity showing the effect of bath application of 10μM KT-5823 while stimulating the ovipositor valves with 1 M sucrose. A 10 min bath application with 10μM KT-5823 resulted in an increase in the cessation of opener muscle activity (C, control, T, test, W, wash).

In summary, NO was found to be involved in the modulation of all 4 chemicals used in the analysis. Increases and decreases of NO levels within the terminal abdominal ganglion, both significantly influenced the cessation of the digging rhythm in response to these chemicals (Fig 6.15A). Moreover, NO was found to modulate the digging rhythm response to sucrose input via a NO/cGMP-PKG signalling pathway, whereas NO seems to modulate the digging rhythm response to NaCl via a NO/cGMP-independent signalling pathway (Fig. 6.15B).

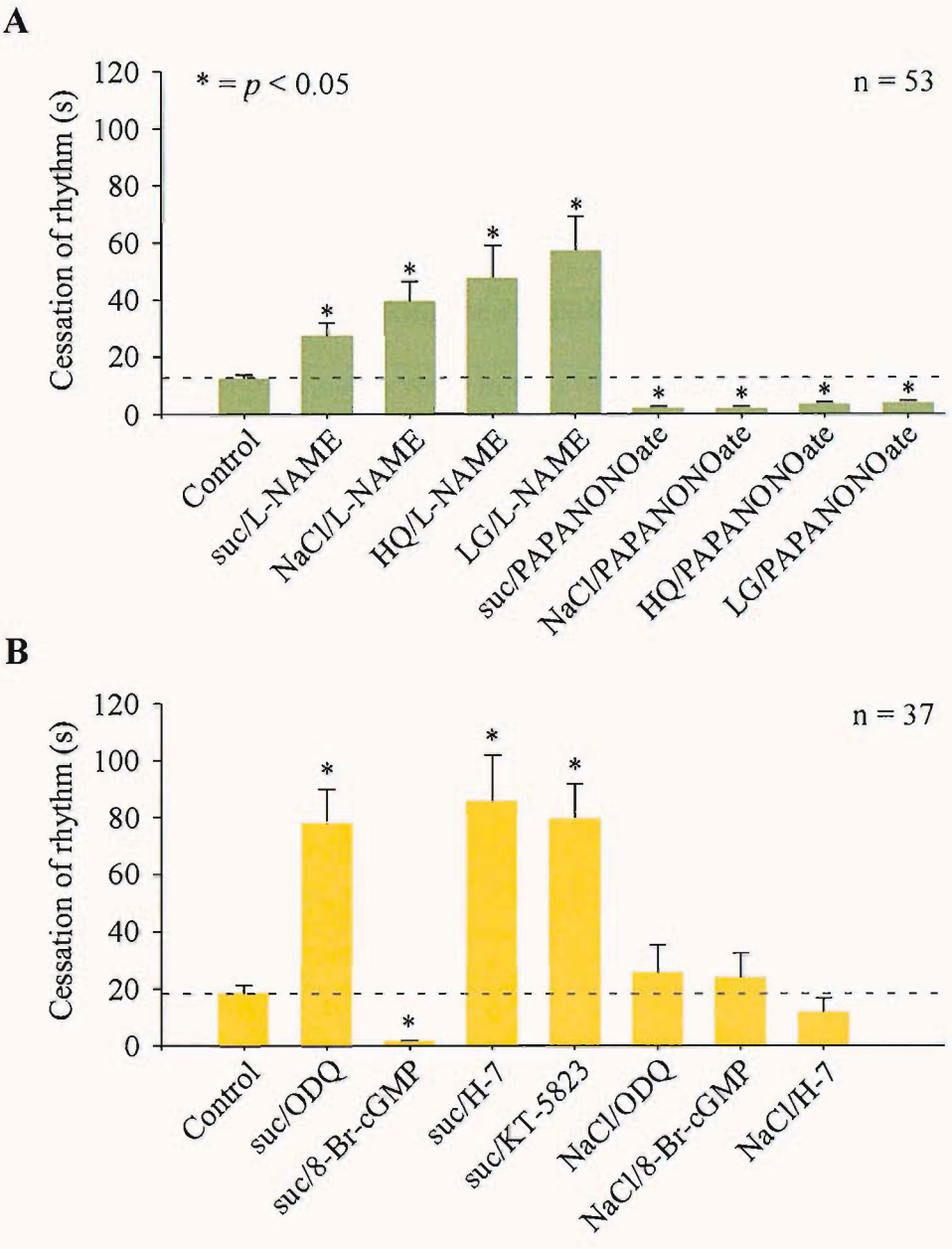



**Figure 6.15. Summary showing the role of NO in the behavioural modulation to chemicals, and the cGMP-dependent and independent behavioural modulation to sucrose and NaCl respectively. A.** Bath application of the terminal abdominal ganglion with L-NAME and PAPANONOate, significantly increased and decreased the cessation of the digging rhythm in response to sucrose, NaCl, HQ and LG. **B.** Inhibiting the molecular targets of NO, sGC and altering levels of cGMP respectively resulted in a significant increase and decrease in the cessation of the digging rhythm respectively, in response to sucrose. Moreover, decreasing PKG resulted in a significant increase in the cessation of the digging rhythm, demonstrating that NO modulates behavioural responses to sucrose via a sGC/cGMP-PKG dependent pathway. Increasing and decreasing levels of sGC, cGMP and PKG did not significantly alter the behavioural responses to NaCl, suggesting that NO modulates behavioural responses to NaCl via a NO/cGMP independent pathway.

### 6.3g Does the cycle frequency of the digging rhythm preceding chemical stimulation influence the duration of cessation of the rhythm?

Chapter 5 demonstrated the involvement of NO in the modulation of the oviposition digging rhythm. NO significantly increased the cycle frequency of the digging rhythm, conversely blocking the synthesis of NO and inhibiting its molecular target resulted in a significant decrease in the cycle frequency. This creates the possibility that the cycle frequency preceding chemical stimulation has an effect on the

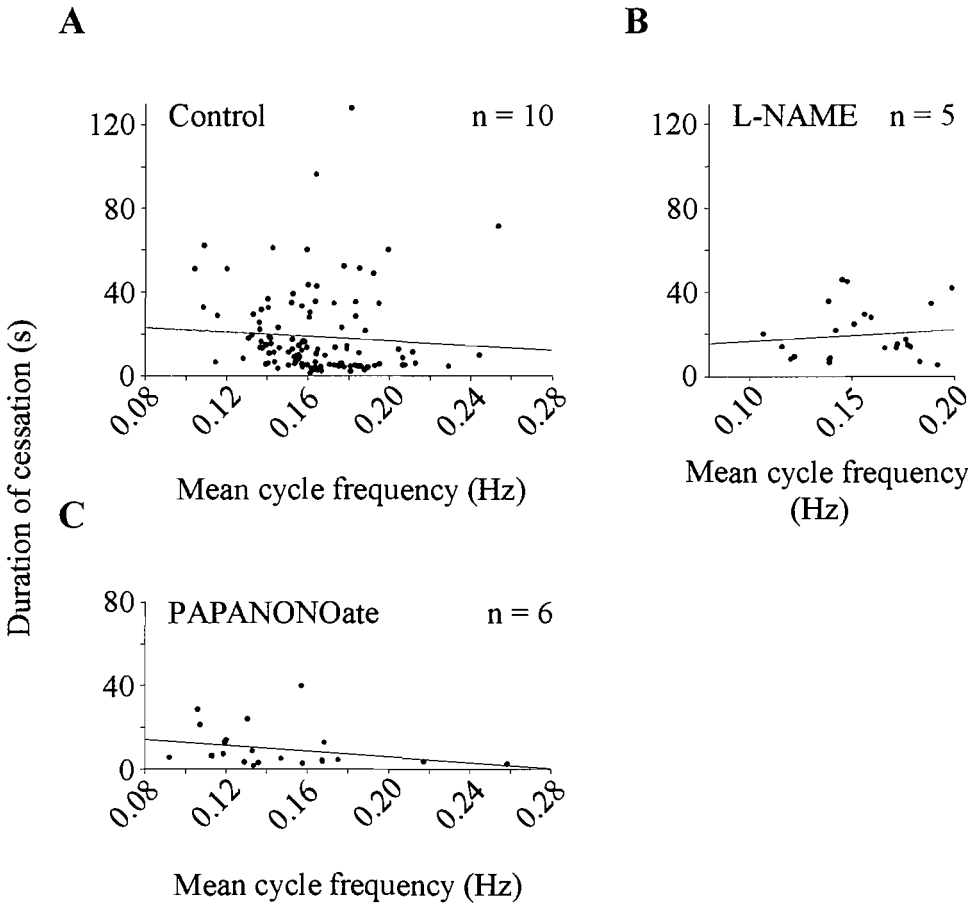



**Figure 6.16. The effect of the mean cycle frequency preceding NaCl stimulation on the cessation of the digging rhythm. A.** Preceding cycle frequency showed a weak negative correlation (r = -0.07) and therefore a weak association with the cessation of the digging rhythm, when the ovipositor valves were chemically stimulated. **B.** When the terminal abdominal ganglion was bathed with 20mM L-NAME and chemically stimulated there was a weak positive correlation (r = 0.11) between preceding cycle frequency and the cessation of the digging rhythm. **C.** Bathing the terminal abdominal ganglion with 0.2mM PAPANONOate while repeatedly stimulating the ovipositor valves with 250mM NaCl, also showed no relationship between preceding cycle frequency and the cessation of the digging rhythm (r = -0.27). This suggests that the cessation of the digging rhythm when the

ovipositor valves were chemically stimulated, was independent of the cycle frequency of the digging rhythm preceding chemical stimulation.

cessation of the digging rhythm. This association was tested for both NaCl and sucrose effects on the digging rhythm during L-NAME and PAPANONOate application.

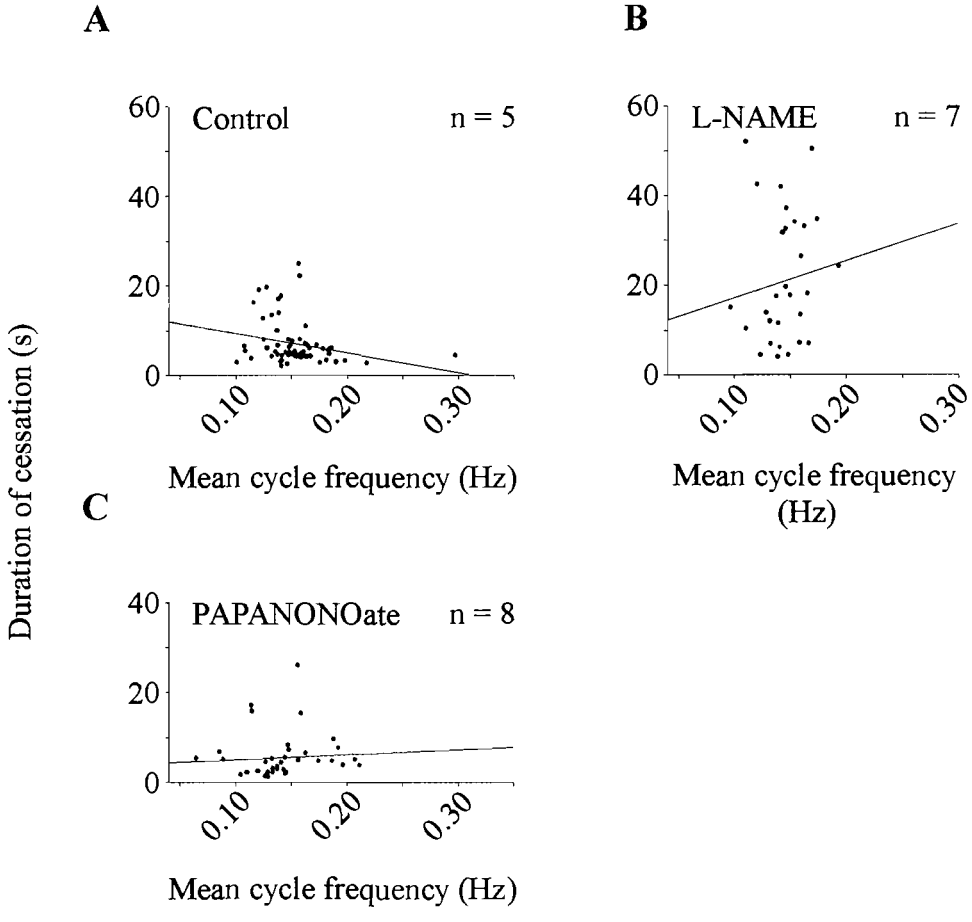



**Figure 6.17. The effect of the mean cycle frequency preceding sucrose stimulation of the ovipositor valves. A.** Preceding cycle frequency showed a weak negative correlation (r = - 0.25) and therefore a weak association with the cessation of the digging rhythm when the ovipositor valves were chemically stimulated. **B.** When the terminal abdominal ganglion was bathed with 20mM L-NAME and the ovipositor valves chemically stimulated, a weak positive correlation (r = 0.12) between preceding cycle frequency and the cessation of the digging rhythm was observed. **C.** Bathing the terminal abdominal ganglion with 0.2mM PAPANONOate while repeatedly stimulating the ovipositor valves with 1 M sucrose, also showed no relationship between preceding cycle frequency and the cessation of the digging rhythm (r = 0.071). This again showed that the cessation of the digging rhythm when the ovipositor valves were chemically stimulated was independent of the cycle frequency of the digging rhythm preceding chemical stimulation.

No correlation was found between cycle frequency preceding chemical stimulation and the duration of cessation of the digging rhythm for NaCl control (correlation coefficient, r = -0.07), 20mM L-NAME-treated (r = 0.11) and 0.2mM PAPANONOate-treated (r = -0.27) experiments (Fig. 6.16A, B, C).

Similarly, no correlation between cycle frequency preceding chemical stimulation and the cessation of the digging rhythm was found for sucrose control (r = -0.25), L-NAME-treated (r = 0.12) and PAPANONOate-treated (r = 0.071) experiments (Fig. 6.17A, B, C). These results show that the cessation of the digging rhythm was due to chemical stimulation and dependent on the presence or absence of NO, and not correlated with the cycle frequency preceding chemical stimulation.

## 6.4 Discussion

It has been shown that manipulating levels of NO in the terminal abdominal ganglion modulates the motor output of the digging rhythm when the ovipositor valves are stimulated with different chemicals. Chemical stimulation with any of the 4 chemicals used in the analysis (sucrose, NaCl, HQ and LG) stopped the digging rhythm. NO synthesis in the terminal abdominal ganglion was decreased by bath applying the NOS inhibitor L-NAME to the terminal abdominal ganglion. This increased the duration for which the digging rhythm stopped. Increasing NO levels by bath applying the NO donor PAPANONOate resulted in a significant reduction in the cessation of the rhythm when the ovipositor valves were stimulated with the 4 chemicals tested. These results show that NO is involved in the modulation of all 4 chemical stimuli tested.

It has also been shown that NO modulates the responses to 2 of 4 chemicals (NaCl and sucrose) via different signalling pathways. Bath applying the cGMP analogue and the sGC inhibitor, 8-Br-cGMP and ODQ, respectively, had no statistical effects on the cessation of the digging rhythm in response to NaCl. Moreover, bath application of the generic protein kinase inhibitor H-7, resulted in no significant increase in the duration of cessation of the digging rhythm when the ovipositor valves were stimulated with NaCl. Conversely, bath application of 8-Br-cGMP and ODQ, resulted in significant decreases and increases respectively, in the cessation of the digging rhythm when the valves were stimulated with sucrose. Repetitive stimulation of the ovipositor valves with sucrose while bath applying H-7 to the terminal abdominal ganglion resulted in a statistically significant increase in the cessation of the rhythm compared to controls. Bath application of the specific PKG inhibitor KT-5823 also resulted in statistically significant increases in the cessation of the digging rhythm when the ovipositor valves were repeatedly

stimulated with sucrose. These results clearly show that NO has the ability to modulate different chemical stimuli through different signalling pathways. NO modulates the behavioural responses to NaCl via a cGMP/PKG-independent pathway while modulating the responses to sucrose via a NO-cGMP/PKG-dependent pathway.

### 6.4a The role of NO in the modulation of contact chemosensory related behaviours

In invertebrates, NO has been shown to have a clear role in modulating behavioural responses to contact chemosensory input (Kobayashi *et al.* 2000). Most work to date has focused on simple feeding networks, such as those in molluscs (Kobayashi *et al.* 2000). In molluscs, transient, rhythmic increases in NO levels are found in the buccal ganglion when the mouthparts of the pond snail, *Lymnaea stagnalis* are stimulated with 100mM sucrose (Kobayashi *et al.* 2000). Sucrose stimulation of the mouthparts is known to initiate rhythmic movements of the mouthparts (Kemenes *et al.* 1986) and increases in NO levels within the buccal ganglion are accompanied with an initiation of feeding (Kobayashi *et al.* 2000). Pharmacological evidence for the role of NO in the initiation of feeding comes from the effects of L-NAME application to the buccal ganglion upon sucrose stimulation of the mouthparts (Kobayashi *et al.* 2000). Following feeding initiation, L-NAME application to the buccal ganglion resulted in a cessation of the rhythmic feeding movements. Similarly in my study, bath application of L-NAME to the terminal abdominal ganglion significantly increased the cessation of the digging rhythm when the ovipositor valves were stimulated with 1 M sucrose. Conversely, increasing NO levels in the terminal abdominal ganglion by bath application of PAPANONOate, while repeatedly stimulating the ovipositor valves with sucrose, resulted in a

significant decrease in the cessation of the digging rhythm. The results of this study support the findings of Kobayashi *et al.* (2000), and provide evidence for a role of NO in the modulation of behavioural responses to contact chemosensory input.

Although NO has been shown to modulate responses to sucrose, there is little evidence to show a role of NO in the behavioural modulation of other contact chemosensory stimuli. In rats, NO has been shown to affect the intake of salt (Roth and Rowland, 1998). When L-arginine was injected into rats, the intake of NaCl was significantly reduced compared to controls (Roth and Rowland, 1998). However, no experiments that decreased levels of NO in the central nervous system were performed in order to confirm that NO mediates the effect. Although no studies have demonstrated a role of NO in the behavioural modulation to bitter chemicals such as HQ, cGMP levels are known to increase in mouse taste buds in response to caffeine and theophylline stimulation (Rosenzweig *et al.* 1999). Although I have not demonstrated that the NO/cGMP-signalling pathway is involved in the behavioural modulation to HQ in this study, a role for NO in modulating the responses to HQ was demonstrated. Similarly, Rosenzweig *et al.* (1999) mimicked the effect of caffeine and theophylline (increasing cGMP levels within mouse taste buds) by bath applying NO. This suggests that the NO/cGMP signalling pathway may be involved in the transduction process of bitter tastes in vertebrates, but also raises the possibility that NO modulates the behavioural responses to HQ in locusts, via a NO/cGMP-signalling pathway. Although there is evidence of a role for NO in modulating the behavioural responses to sucrose, salt and bitter tasting compounds such as theophylline, caffeine and HQ, to date there is no evidence for a role of NO in modulating the behavioural responses to amino acids. This is therefore the first time that NO has been shown to modulate behavioural responses to a range of different chemical stimuli, including sucrose, NaCl, HQ and LG.

### 6.4b Molecular targets of NO during the modulation of contact chemosensory input

Studies on the blowfly, *Phormia regina* have shown the involvement of NO in the modulation of the TRC response to sucrose (Murata *et al.* 2004). Decreasing levels of NO by extracellularly introducing the NO scavenger, PTIO into the TRC's was found to suppress the number of action potentials evoked from the sucrose responsive TRC upon sucrose stimulation (Murata *et al.* 2004). Conversely, introducing the NO donor, NOC-7 into the TRC's was found to induce action potentials in the sugar responsive TRC, suggesting that NO may be involved in the transduction of sucrose stimuli in insects (Nakamura *et al.* 2005). Although Nakamura *et al.* (2005) suggest that NO acts via a sGC/cGMP signalling pathway to modulate the taste receptor response to sucrose stimulation, they provide no experimental evidence to support this. The results of this study show for the first time that responses to sucrose stimulation in insects are modulated by the NO/cGMP signalling pathway.

Although there are no other examples that support the role of sGC and cGMP in modulating the behavioural responses to sucrose, there is evidence to suggest a potential role of cGMP in the detection of sucrose in the oral cavity of vertebrates. In the circumvallate taste bud cells of rats, a rapid and transient increase in intracellular levels of cGMP is observed in response to sucrose stimulation (Krizhanovsky *et al.* 2000). When circumvallate taste cells were isolated from the rat and stimulated with sucrose, a radioimmunoassay for cAMP and cGMP showed significant increases in the levels of cGMP in circumvallate taste cells, compared to unstimulated circumvallate taste cells and non-sensory epithelial cells. Similarly, when circumvallate taste cells were stimulated with sucrose in the presence of a specific cGMP-phosphodiesterase (PDE) inhibitor, increases in intracellular levels of

cGMP were not observed (Krizhanovsky *et al.* 2000). The above results suggest that cGMP may have a role to play in the transduction of sucrose stimuli.

In comparison to the results of this study, bathing the terminal abdominal ganglion with the membrane permeable analogue of cGMP, 8-Br-cGMP, while stimulating the ovipositor valves with sucrose decreased the duration of cessation of the digging rhythm. These results suggest that increases in NO (and hence cGMP) levels in the terminal abdominal ganglion, decrease the aversive chemosensory input onto the rhythm (decrease duration of cessation of the digging rhythm) and function to maintain oviposition digging, for example, when a suitable oviposition substrate is encountered. Indeed, Schuppe *et al.* (pers. comm.) have demonstrated that the presence of NO around the sensory neurons of basiconic sensilla of locusts, markedly affects the sensory neurons response to NaCl stimulation. Stimulation of the sensory neurons with NaCl using the tip recording technique (Hodgson *et al.* 1955) evokes action potentials from the sensory neurons which are then propagated to the central nervous system. When the sensory neurons were bathed with PAPANONOate, the frequency of action potentials evoked from the sensory neurons when stimulated with NaCl significantly decreased. As the frequency of action potentials increases with increasing concentration of NaCl, and therefore the aversiveness of NaCl increases (Newland, 1998; Rogers and Newland, 2000), this suggests that the presence of NO in either the periphery or the terminal abdominal ganglion as shown in this study, decreases aversive input of a chemical stimulus, into the central nervous system. Moreover, Schuppe *et al.* (pers. comm.) also showed that increased levels of cGMP in the sensory neurons had no effect on the frequency of action potentials when the sensory neurons were stimulated with NaCl, providing further support for behavioural responses to NaCl being modulated via an NO/cGMP-independent pathway.

### 6.4c The role of protein kinase in the modulation of contact chemosensory input

In the honeybee, *A. mellifera*, PKA modulates the gustatory responsiveness to sucrose (Scheiner *et al.* 2003). Gustatory responsiveness in *A. mellifera* was measured by calculating the probability that individuals would produce a proboscis extension reflex (PER) when the mouthparts were stimulated with 6 concentrations of sucrose. High gustatory responsiveness was indicated by a PER to all 6 concentrations of sucrose, whereas low gustatory responsiveness was indicated by a PER to 30% or 60% sucrose solution only (Scheiner *et al.* 2003). Injecting an activator of PKA, 8-Br-cAMP, into the thorax of *A. mellifera* increased the gustatory responsiveness, with gustatory responsiveness increasing with time after injection.

As behavioural responses to NaCl rely on the initial extracellular detection of $Na^{2+}$ ions by the dendrites of sensory neurons located within the taste receptors, a potential target for NO in the modulation of behavioural responses to NaCl contact chemosensory input is sodium channels (Hammarström and Gage, 1999). For example, in rat hippocampal neurons, the presence of exogenous NO affects the opening of sodium channels. Whole cell recordings were made of hippocampal neurons, measuring the whole cell inward sodium current. In the presence of the NO donors SNP and SNAP, the amplitude of the whole cell current increased by between 60-80% of its original control value (Hammarström and Gage, 1999), indicating that the presence of exogenous NO, has the ability to modulate the behaviour of sodium channels. This suggests that in contrast to the modulation of the digging rhythm in response to sucrose stimulation of the ovipositor valves, NO may be modulating the digging rhythm response to NaCl stimulation by having a direct effect on sodium

channels of individual neurons at the level of the terminal abdominal ganglion, and hence modulating the motor output of the CPG.

# Chapter 7 – General Discussion

# Chapter 7

# General Discussion

The five experimental chapters of this thesis have demonstrated the importance of taste in the selection of an egg-laying substrate by locusts. Subsequent chapters detail the role of NO in modulating both the oviposition digging rhythm and the response of the rhythm to 4 of 7 chemicals tested in the study. Finally, NO was found to modulate the response of the digging rhythm to 2 different chemicals via different signalling pathways. NO modulates the behavioural responses of the ovipositor valves to sucrose input via a NO/cGMP-PKG-dependent signalling pathway, whereas it modulates the responses to NaCl input via a NO/cGMP-independent signalling pathway.

## 7.1 The effect of chemical type and concentration on egg-laying behaviour

My initial studies that analysed the effect of specific chemicals on different parameters of locust egg-laying behaviour, demonstrated that a degree of behavioural plasticity was present when locusts attempted to lay eggs within a substrate. For example, chemicals within the substrate resulted in a significant reduction in both the number of egg pods laid, and the duration that locusts exhibited egg-laying behaviour. Surprisingly, chemicals at their higher, and therefore most aversive concentrations, did not stop locusts attempting to lay eggs within those substrates. This suggested that aversive contact chemosensory input onto the CPG underlying digging could be over-ridden, a finding also observed by Woodrow (1965). The reason for this behavioural plasticity is unclear, but insects are known to differ in terms of their egg-load and ideal egg-laying substrates may be unavailable in a natural situation (Drost and Carde, 1992). Moreover, high egg-loads in gravid insects are potentially costly and a physiological mechanism that allows egg-laying

to occur, despite the unavailability of a suitable egg-laying substrate, could be of benefit (Sadeghi and Gilbert, 2000). For example, in parasitic wasps, *Venturia canescens*, individuals with high egg loads laid eggs in low quality hosts compared to low egg load individuals (Fletcher *et al.* 1994) that showed discriminatory behaviour and preferentially laid eggs in high quality hosts. This suggests that gravid locusts also vary in egg loads, possibly due to the duration for which individuals are deprived of egg-laying substrates, for example between experiments, and may explain why attempts at egg-laying were observed in substrates containing aversive concentrations of chemicals.

Despite locusts showing no discriminatory ability in terms of egg-laying attempts, locusts were found to make a behavioural choice between suitable and unsuitable egg-laying substrates. Locusts preferentially laid egg pods within substrates containing distilled water treated substrates, compared to substrates containing higher concentrations of chemicals. Moreover, locusts maintained digging behaviour in acceptable substrates, compared to substrates containing higher chemical concentrations. Longer durations of digging were therefore observed in control substrates with significant reductions in digging duration for higher chemical concentrations. This raises the possibility that physiological mechanisms at the level of the central or peripheral nervous system could underlie the choice between suitable and unsuitable egg-laying substrates.

## 7.2 The role of NO in the modulation of chemosensory input

It has been shown that NO modulates behavioural responses to contact chemosensory input. Experiments on isolated abdomens showed that 250mM NaCl, 1 M sucrose, 2 M LG and 100mM HQ acted aversively by significantly increasing the duration of cessation of the digging rhythm, compared to their lower concentrations. Increasing NO levels however, reduced the aversive effect of all of

these chemicals on the digging rhythm, with the duration of cessation of the digging rhythm significantly decreasing, to the extent that in some cases there was no interruption in the digging rhythm following chemical stimulation of the ovipositor valves. On the other hand, the duration of cessation of the digging rhythm significantly increased when NO levels were decreased while the ovipositor was stimulated with NaCl, sucrose, HQ and LG.

These results suggest that NO modulates contact chemosensory input onto the CPG network that underlies digging. Where NO has its effects in a freely behaving animal however, has to date not been resolved, although Ott *et al.* (2001) have shown that one of its molecular targets, sGC, is located in the chemosensory neurons in the periphery. NO has been recently shown to act at the level of the sensory neurons that respond to contact chemosensory stimulation. The frequency of action potentials evoked by stimulating the sensory neurons with NaCl are significantly reduced by the presence of NO compared to control (Schuppe. pers. comm.). Although, as demonstrated in this study, NO can also act at the level of the central nervous system to modify motor output both in the presence (see *Chapter 6*) and absence (see *Chapter 5*) of chemical stimulation of the ovipositor valves. Within the central nervous system the neurons upon which NO exerts its effects to modify the motor output of the digging rhythm are unknown, although the likely targets may be motor neurons as shown by Ott *et al.* (2000).

When an unsuitable substrate is encountered, such as a substrate containing a high concentration of a chemical, levels of NO may decrease leading to the termination of the digging rhythm. Although NO also had different independent effects on the digging rhythm in the absence of chemical stimulation (increases and decreases in cycle frequency with increasing and decreasing levels of NO), there was no evidence that the effects of NO on the digging rhythm itself influenced the response to chemical input. For example, the cycle frequency of the rhythm

preceding chemical stimulation did not influence the duration of cessation of the digging rhythm during chemical stimulation.

This study did not establish the targets of PKG, although targets include the phosphorylation of $K^+$ channels (Moreno *et al.* 2001). Elevations of PKG presumably alter the intracellular environment of neurons via the phosphorylation of proteins that in turn modulates the activity of a neuron, or groups of neurons to modify motor output (Gertsberg *et al.* 2004). PKG could also directly target ion channels such as $K^+$ channels and modulate either the depolarization or repolarization of neurons or groups of neurons and modulate their motor output (Moreno *et al.* 2001).

## 7.3 Signalling pathways involved in modulating behavioural responses to contact chemosensory input

Recently, Wu *et al.* (2005) showed that *Drosophila* deprived of food would actively feed on food treated with quinine, food normally avoided by *Drosophila* that had previously been fed a normal diet. Over expression of neuropeptide F receptor (NPFR 1), a mammalian neuropeptide Y (NPY) receptor homolog, caused normally fed *Drosophila* to feed on quinine treated food (Wu *et al.* 2005) so that normal aversive responses to quinine treated food were abolished. Moreover, simultaneously over expressing 7 genes that encode for insulin-like proteins in NPFR 1 *Drosophila*, led to attenuated feeding on quinine treated food, suggesting that NPY maybe modulated by an insulin-like signalling pathway (Wu *et al.* 2005).

Previous studies have also indicated the involvement of protein kinases in the modulation of behavioural responses to contact chemosensory input. Directly injecting cAMP, an activator of PKA into the honeybee *A. mellifera* has also been shown to increase the probability that a PER reflex was evoked in response to

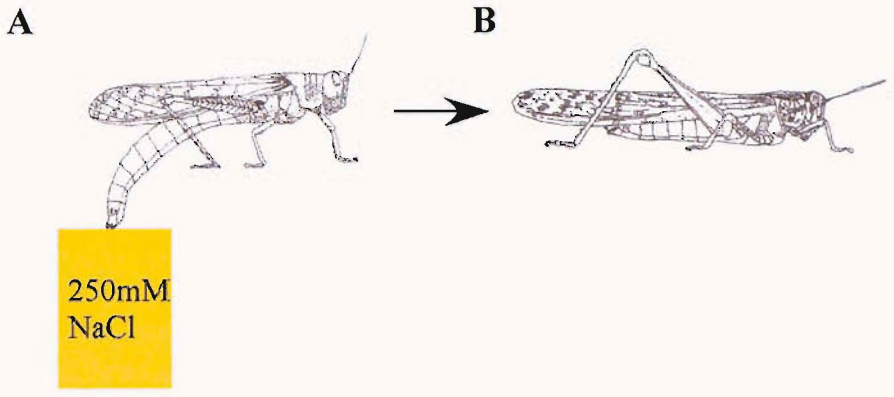
sucrose stimulation of the mouthparts (Scheiner *et al.* 2003). What causes changes in the levels of cAMP, however, have yet to be identified.

The signalling pathways that regulate aversion to noxious chemicals and food appear to be diverse. This study shows that although NO is involved in modulating the behavioural responses to high (noxious) concentrations of 4 chemicals, the molecular targets of NO could vary. It has been shown that NO modulates behavioural responses to NaCl and sucrose via 2 different signalling pathways. The possibility therefore arises that NO has other molecular targets other than cGMP to modulate behavioural responses to other chemicals such as HQ and LG. Further studies are necessary to establish whether NO modulates behavioural responses to other chemicals of behavioural relevance, and whether NO subsequently acts on different molecular targets depending on the chemical used to stimulate the ovipositor valves. Conversely, the NO/cGMP signalling pathway may be more conservative, and high concentrations of many, but not all chemicals, may be modulated via the same signalling pathways.

## 7.4 How does the NO/cGMP-PKG signalling pathway modulate oviposition?

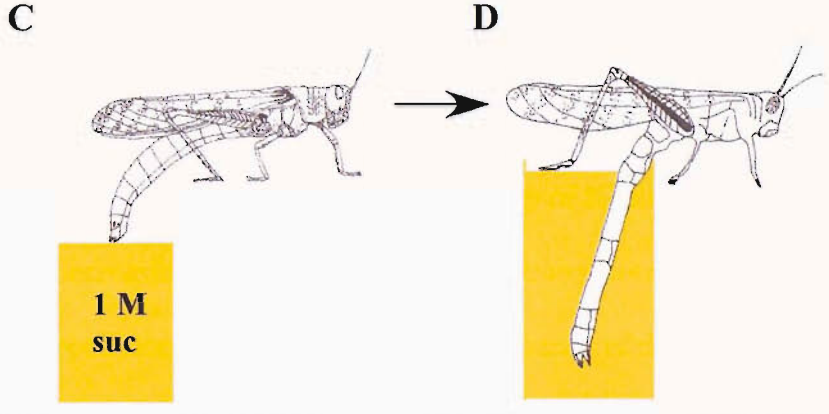
It has been established that NO modulates the behavioural responses to different chemicals using different signalling pathways. Less clear, is how NO and the different signalling pathways through which NO may act, may underlie the choice of egg-laying sites. Chapter 2 showed that locusts laid eggs in 1 M sucrose, but not in 250mM NaCl (Fig. 2.2A, C). This suggests that if locusts are given a choice between a substrate containing 250mM NaCl and 1 M sucrose, locusts would preferentially lay eggs within the substrate containing 1 M sucrose. Based on data presented in chapter 6, on encountering an egg-laying site containing 250mM NaCl, NO levels within the terminal abdominal ganglion would decrease, decreasing the

frequency of the oviposition digging rhythm and also increasing aversive NaCl




Locust samples 250mM NaCl with its ovipositor valves. NO levels in the terminal abdominal ganglion decrease, the cycle frequency of the oviposition digging rhythm decreases and digging stops.

250mM NaCl is rejected as an egg-laying site and the locust continues its search for a suitable egg-laying site.




Locust encounters a substrate containing a 1 M concentration of sucrose. NO levels in the terminal abdominal ganglion increase and target sGC, increasing levels of cGMP. cGMP then activates PKG. NO, via the sGC/cGMP-PKG signalling pathway increases the cycle frequency of the oviposition digging rhythm and digging is maintained in the presence of 1 M sucrose.

Oviposition digging continues as the substrate is selected for egg-laying and the abdomen extends into the substrate in order to lay eggs at depth.

**Figure 7.1. The role of the NO/cGMP-PKG signalling pathway in the selection of egg-laying sites. A.** On encountering a substrate containing 250mM NaCl, NO levels within the terminal abdominal ganglion decrease, significantly decreasing cycle frequency and stopping the digging rhythm. **B.** Substrates containing 250mM NaCl are rejected and locusts maintain their search for a suitable egg-laying site. **C.** When a substrate containing 1 M sucrose is sampled with the ovipositor valves, NO levels in the terminal abdominal ganglion increase, targeting sGC to elevate levels of cGMP, which in turn activates PKG significantly increasing the cycle frequency of

the digging rhythm. **D.** The oviposition digging rhythm is maintained in the presence of 1 M sucrose and the substrate selected as a suitable egg-laying site.

chemosensory input into the central nervous system (Schuppe. pers. comm), eventually stopping the digging rhythm (Fig. 6.1B, Fig. 7.1A, B).

After rejecting the substrate containing 250mM NaCl, locusts would continue to search for a suitable egg-laying substrate. On encountering a substrate containing 1 M sucrose, locusts would initially sample the substrate. Experiments using isolated abdomen showed that 1 M sucrose can act aversively by stopping the oviposition digging rhythm for significantly longer durations compared to low concentrations (Fig. 4.3A). In terms of selecting and egg-laying substrate however, NO levels within the terminal abdominal ganglion would increase. Elevations in the levels of NO would activate the enzyme sGC which in turn would catalyse GTP to cGMP, resulting in a significant increase in intracellular cGMP levels. cGMP would then activate PKG which could increase the activity of $K^+$ channels (Reddy, 2006), possibly decreasing the duration taken for neuronal components of the oviposition CPG to repolarise and increase the cycle frequency of the digging rhythm (Fig. 5.3A, B, C, D; Fig. 5.4A, B, Fig. 7.1C). Aversive sucrose chemosensory input into the terminal abdominal ganglion would be overridden and the digging rhythm maintained (Fig 6.4A, B). Locusts would maintain digging and therefore select a substrate containing 1 M sucrose as an egg-laying substrate (Fig. 7.1D).

This study shows pharmacological evidence for a role of the NO/cGMP-PKG signalling pathway in modulating the oviposition digging rhythm. Despite this evidence, it is not clear where in the terminal abdominal ganglion NO is acting. Müller and Bicker (1994) showed that interneurons within the terminal abdominal ganglion showed both NOS and NADPHd activity, although it was noted that only interneurons showed a capability of producing and releasing NO. A total lack of

NOS and NADPHd activity was observed for both motor and sensory neurons, suggesting that NO acts as a signalling molecule primarily at the level of the central nervous system (Müller and Bicker, 1994).

Recent studies that compare the effectiveness of NOS-immunocytochemistry and NADPHd-histochemistry however, have shown that there is a distinct presence of NADPHd-positive fibres within the ventral association centres (VAC's) within the ventral neuropiles of the terminal abdominal ganglion (Bullerjahn and Pflüger, 2003), which consist predominantly of mechano- and chemosensory fibres (Burrows and Newland, 1994). These findings provide additional evidence that NO modulates the behavioural responses to contact chemosensory input at a very early stage of chemosensory processing, and that aversive chemosensory input (from the peripheral nervous system) onto the CPG that underlies oviposition digging can be manipulated by increasing or decreasing NO (Schuppe. pers. comm.). For example, decreasing NO levels in the sensory neurons of basiconic results in a significant increase in the number of action potentials propagated to the central nervous system (Schuppe. pers comm). Similarly, reducing NO levels in the terminal abdominal ganglion significantly increased the duration for which the digging rhythm stopped when the ovipositor valves were stimulated with 1 of 4 chemicals. This results of these 2 studies, compared with what is known about the location NOS-positive staining in the terminal abdominal ganglion (VAC's and sensory neuropiles), suggests that NO mediates communication between the peripheral and central nervous system and modulates sensory input onto the CPG that underlies oviposition digging. Manipulating NO levels in the terminal abdominal ganglion therefore significantly affects the intensity of the chemosensory stimulus onto the oviposition digging CPG, and modulates the behavioural output (duration for which the digging rhythm stops) accordingly.

Although we have an idea of the types and locations of neurons and regions of the central nervous system that synthesise and release NO, the locations of neurons that synthesis PKG are not known. Immunohistochemical studies using the terminal abdominal ganglion would allow identification of the neurons that synthesis PKG in response to NO increases, and allow a more detailed understanding of how NO synthesizing neurons communicate with target neurons.

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