PROJECT REPORT No. 338

UNDERSTANDING THE HORMONAL PHYSIOLOGY OF REPRODUCTION IN SLUGS AS A BASIS FOR POTENTIAL NOVEL CONTROL METHODS

MAY 2004

Price £4.50

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UNDERSTANDING THE HORMONAL PHYSIOLOGY OF REPRODUCTION IN SLUGS AS A BASIS FOR POTENTIAL NOVEL CONTROL METHODS

by

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This is the final report of a 48-month project that started in January 2000. This work was funded by the HGCA with a grant of £127,345 (Project 2218).

CONTENTS

ABSTRACT

The overall scientific aim of the project was to investigate the physiology and biochemistry of terrestrial molluscan endocrine systems regulating development (including the processes of protandry, courtship and mating behaviour) in order to evaluate their potential as slug pest management targets. There were two major scientific objectives:

i) the investigation of the endogenous hormones and neuropeptides that participate in the endocrine control of reproduction and development in terrestrial pulmonate gastropods with the view to identifying potentially effective and precise targets for the development of novel molluscicides, and

ii) the exploration of the potential of specific peptides as novel slug control methods and devising novel effective delivery systems for them.

The key-results from the project (described in detail under the Summary and the Technical Detail sections that follow) were promising, as:

i) the project highlighted certain molecules as potential candidates for the development of novel pest slug control agents, and

ii) it explored further for effective and inexpensive delivery systems for such novel control methods in the field.

SUMMARY

The key messages emerging from the project, as well as the relative conclusions and implications for the levy payers are as follows:

i) During the 1st year of the project **we designed and applied a specific experimental protocol for culturing terrestrial slugs under laboratory conditions, and in particular the grey field slug *Deroceras reticulatum***. The establishment of a breeding unit was an essential tool for the project as it ensured a) continuous access throughout the calendar year without being dependent on either the weather conditions and/or the slug abundance in the field, b) access to reliable biological material (i.e. slugs of known physiological status and age, therefore contributing to reduced biological variability for our assays) during the whole project.

ii) A **thorough literature investigation of the current state of knowledge on the reproductive endocrinology of terrestrial pulmonate gastropods (i.e. terrestrial slugs and snails)** was performed during the 1st year of the project. This research review was crucial in order to identify the endocrine system(s) controlling the development and reproduction in these animals, and consequently prioritise the key targets for further investigation.

iii) Following the identification of the prospective targets for endocrine disruption in terrestrial slugs, we concentrated our research on a particular hormone, the Dorsal Body Hormone, which participates in the regulation of the female reproductive activity (including egg-laying) of terrestrial slugs and snails. During the 2nd year of the project we tackled the question of whether **the Dorsal Body Hormone** in terrestrial slugs **is an ecdysteroid**, as certain previous research indicated, **and we showed that this is unlikely**.

iv) Next, following the results indicating the absence of any ecdysteroids in the central nervous system of terrestrial slugs, we directed our research towards the investigation of the peptidic or proteinaceous nature of the Dorsal Body Hormone. To this end, **we developed an experimental protocol based on the technique of Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI/TOF-MS)** during the 2nd year of the project. We successfully applied this protocol in order to show that **MALDI/TOF-MS can be used directly on slug nervous tissue and can produce reliable results**.

v) Our research on the Dorsal Body Hormone continued by **establishing a bioassay** (measuring the incorporation of ^{14}C in polysaccharide synthesis in *in vitro* cultures of albumen gland) **that can be used to detect Dorsal Body Hormone activity**. Subsequently, we were able to show that there are at least two polysaccharide synthesis stimulating factors (POL-SFs) (one of which is the Dorsal Body Hormone) in the central nervous system of terrestrial slugs. The POL-SFs appear to be secreted in the central nervous system of the slugs during the female phase of the reproductive maturity of the animals, except when the slugs enter the latest stages of their reproductive maturation. The **establishment of this bioassay** was **crucial** as it **can be used in further future**

research in order to a) **screen for biological activity of the Dorsal Bodies Cells**, b) **investigate the inhibition of the Dorsal Body Hormone function**, and c) **evaluate potential endocrine-disruptive molecules of the female reproductive maturation in terrestrial slugs**.

vi) Throughout the 3rd year of the project we also carried out several experimental trials evaluating the potential of a specific lectin, in particular the *Galanthus nivalis* agglutinin (GNA: showdrop lectin) as a carrier of biologically active molecules against slugs. The results obtained demonstrated that **GNA can indeed function as a carrier molecule as it can be transported into the haemolymph of the animals following oral ingestion**. This was an important key result as it opened up the **opportunity to look into the possibility of using fusion proteins**, in particular those with GNA as a carrier molecule, **as a new technology for the development of novel slug baits**, etc.

vii) After we obtained the above result, we decided to look into the practical value of the prototype fusion protein (formulated in CSL in collaboration with the university of Durham) in which GNA was linked to an insect neuropeptide *Manduca Sexta* allatostatin (Manse-AS) that was known to act as a potent feeding inhibitor in insects. Nevertheless, its potential as a terrestrial slug control agent is questionable following our inability to a) unequivocally demonstrate the presence of this particular neuropeptide in the CNS of slugs, and b) to detect any biological activity of this particular neuropeptide (Manse-AS) on the feeding of slugs (experimental trials performed during the 3rd year of the project).

viii) Additionally, during the 3rd year of the project, **we assessed the egg-laying activity of the grey field slug** *D. reticulatum* in the lab **with the view to obtaining as much information as possible in order to design precise bioassays to be used for the evaluation of certain neuropeptides as potential reproductive and/or egg-laying inhibitors**. The egg-laying activity of the grey field slug was assessed as highly complex and variable, therefore indicating that *D. reticulatum* is rather opportunistic in its egg-laying behaviour. Nevertheless, **the data accumulated provided us with an important basis upon which we developed a bioassay that we used to measure reliably the inhibition of the reproductive maturity or of the egg-laying production in pest slugs** (see below).

ix) During the final year of the project we assessed the potential of a known reproductive inhibitor in freshwater snails, the *Lymnaea* neuropeptide Y (LyNPY), as an egg-laying inhibitor in terrestrial slugs. Results showed that **LyNPY can indeed exert an inhibitory effect on the egg-laying activity of *Deroceras reticulatum***, since daily injections (at the very low concentration of 1μM) of the neuropeptide resulted to reduced egg production by the slugs (by 40% as compared with the control slugs).

x) Our research on possible reproductive inhibitors of terrestrial slugs continued by investigating the potential of the neuropeptide FMRFamide, for which previous research indicated that it might function as a regulator of reproduction in pulmonate gastropods by participating in the inhibition of Dorsal Body Hormone (experimental trials performed during the final year of the project). Our

results indicated that **FMRFamide could also have a potential as a reproductive regulator of the grey field slug**, as **weekly injections** (at the concentration of 0.1mM) of the **neuropeptide appeared to delay the rate of their reproductive maturity**, possibly by inhibiting the function of the main female hormone (i.e. Dorsal Body Hormone). Nevertheless, further research is needed in order to quantify the possible relationship between this neuropeptide and the main female gonadotropic hormone in terrestrial slugs.

xi) Finally, during the final year of the project, we focused on naturally occurring toxins because it is widely accepted that they are good candidates for the development of novel and highly efficient pesticides, due to their high specificity and the fact that they target crucial parts of the biochemical pathways and mechanisms at the cell level. We concentrated our research on toxins originating in the molluscan phylum, and we selected one (namely TxIA conotoxin) based on its high specificity for molluscan targets (including terrestrial snails) and the fact that it shows no effects on either other invertebrates (e.g. arthropods) or vertebrates (e.g. rats). **It was shown that TxIA has adverse effects** (i.e. inhibition of feeding activity, decrease of weight, mortality) **on the grey field slug**, *D. reticulatum*, **in particular during the initial 3-4 days following its administration** (by injections into the haemocoel of the slugs). The exact physiological mechanisms underlying these effects are not clear, and further research is needed in order to refine the exact function and the stability of this particular toxin.

xiv) During the 4 years of this project there has been some technology transfer towards a) the farming community (Crops magazine - June 2001 issue; Crops magazine – June 2003 issue; Pesticide outlook- June 2001; Cereals - June 2004; Monitoring events – February 2000 - February 2001 - October 2002 - September 2003), and b) the scientific community (Meeting of International Organisation on Biological Control, Working Sub-Group on Slugs and Snails - Lyon, March 2001;Seventh International Congress on Medical and Applied Malacology - Philippines, October 2002; BCPC conference on Slugs and Snails in Agriculture – Canterbury, September 2003; Publication of the review – Acceptance date 2/09/2003).

TECHNICAL DETAILS

A. Introduction

A. 1. Terrestrial slugs as pests

Terrestrial slugs belong to the Phylum Mollusca, Class Gastropoda, subclass Pulmonata. Interest in terrestrial slugs has increased in recent years mainly because several slug species [for example *Arion hortensis* agg., *Deroceras (=Agriolimax) reticulatum*, *Tandonia (=Milax) budapestensis*] are important agricultural and horticultural pests (Glen and Moens, 2002). As a result of their water-permeable integument, slugs are restricted to live in habitats where conditions remain moist for most of the time, for example heavy soils and dense vegetation. They are most abundant on heavy clay and silt soils because these tend to remain moist, and the soil structure allows easier movement of slugs through the soil. In natural habitats slugs feed on a wide range of the available plant material, usually herbaceous plants and grasses. Most slugs exhibit a preference for certain food plants. In the case of *D. reticulatum* these are mainly Leguminosae, Cruciferae and Compositae.

The main slug damage in relation to agricultural crops occurs in oilseed rape, potatoes and in particular winter wheat. Regarding the latter, slugs hollow out wheat seeds soon after sowing, eating the embryo first and thus preventing germination. This type of damage is one of the most important causes of failure of the crop. In addition, slugs may cause serious damage to wheat plants by feeding on the growing coleoptiles, young shoots and leaves. In general, young plants (seedlings) are much more susceptible to damage than established plants. Damage to seedlings can be controlled by slug pellets, however the damage caused to buried seeds is more difficult to prevent. The most serious damage to winter wheat occurs soon after sowing, when slugs hollow out the embryos of the seeds or eat through the base of the stems of seedlings. As a result, the plants may fail to germinate or be killed shortly after emergence. The resulting gaps in the growing crop allow colonisation by unwanted weed species, as well as reducing overall yield. Previous surveys (Strickland, 1965; Hunter, 1969; Stephenson and Bardner, 1976) have indicated that up to 2.2% of the wheat crop (approx. 350,000 tonnes) can be lost as a result of the damage caused by slugs. In financial terms the damage to winter wheat in terms of lost production has been estimated to be in the region of £2.69m annually. In addition, the annual cost of slug control measures is estimated to be about £8.69m for wheat (Port and Port, 1986).

Moreover, a wide range of horticultural crops are attacked by slugs. The major problem to these crops is contamination by faeces and transfer of fungal spores from rotting to healthy plants. Feeding and soiling with faeces and slime results in a lowering of the quality and size of the harvest as well as destruction of crops in fields and gardens. The most extensively damaged horticultural crops are lettuce and Brussels sprouts (Port and Ester, 2002). Moreover, plant pathogenic organisms (bacteria and fungi) may establish themselves in plant tissues that have been damaged by slugs (Godan, 1983). Furthermore, recent changes in UK farming practice, such as the more extensive use of straw incorporation methods, have created even more ideal conditions in which slug populations can multiply.

In addition to the above, slugs can act as vectors of helminth parasites of domestic and wild mammals and birds. These include the nematodes *Acanthocephalus cantonensis*, which can also cause eosinophilic meningo-encephalitis in humans, and *Acanthocephlus costaricensis*, which causes human abdominal angiostrongyliasis (South, 1992). Furthermore, slugs may also be capable of acting as vectors of various plant diseases (see articles cited in South, 1992).

A. 2. Current slug control methods: problems and efficacy

The history of chemical slug control starts as early as 1439 when dry dressings (e.g. salt) were first used in order to irritate the animals so that they would eventually desiccate (Konrad von Megenberg cited in South, 1992). Since then a great many other substances have been used for slug control (extensive literature cited in South, 1992; Henderson and Triebskorn, 2002; Bailey, 2002), but currently, only three molluscicides are approved for the control of snails and slugs in UK:

- Metaldehyde (2,4,6,8-tetramethyl-1,3,5,7,-tetroxocane), which is an irritant poison that, in slugs, causes excessive mucus secretion, leading to death by dehydration. Metaldehyde is mainly used in field crops, vegetables, fruit crops, ornamentals and glasshouse crops.

- Methiocarb [4-(methyl-thio)-3,5-xylyl methylcarbamate], which is a conventional carbamate insecticide with neurotoxic action, and is mainly used in field crops, vegetables, blackcurrants and strawberries. Methiocarb is the most widely used molluscicide in cereals, and in 1990 it was applied to 79% of the total cereal crop acreage in which molluscicides were used (Davis *et al.,* 1991).

- Thiodicarb (3,7,9,13-tetramethyl-5,11-dioxa-2,8,14-2,8,14-tritia-4,7,9,12-tetra-azapentadeca, 12-diene-6,10-dione) that was introduced commercially in the late 1980s.

The above chemicals are referred to in the literature as "highly specific molluscicides", however, as stated above, they can also affect non-target organisms. A comparison of metaldehyde and methiocarb as slug poisons, based on experiments performed under laboratory conditions suggests that both chemicals act quite effectively against slugs, with methiocarb being slightly more effective. Field trials also indicated that there are similar levels of efficacy (Port and Port, 1986). Field evaluations show thiodicarb to be as effective as methiocarb (Ferguson *et al*., 1995). Laboratory trials suggest that thiodicarb may give better control in wet conditions and methiocarb better in dry conditions (Ferguson *et al*., 1995).

One of the main problems associated with the use of these molecules for slug control is the inadvertent poisoning of non-target organisms. Additionally, both metaldehyde and methiocarb have been implicated in the deliberate poisoning of wildlife. Metaldehyde is toxic to vertebrates and several instances of poisoning have been reported in dogs, cats, sheep and poultry (Homeida and Cooke, 1982; Richardson *et al*., 2003). Methiocarb is toxic to beneficial invertebrates that are important predators of other pests. Poisoning by methiocarb pellets has been reported in invertebrates such as earthworms (Bieri *et al*., 1989) and carabid beetles (Buchs *et al*., 1989) and also in vertebrates such as sheep (Giles *et al*., 1984), cats and dogs (Studdert, 1985). In addition, several wild mammals (e.g. field mice, hares and hedgehogs) and birds (e.g. pheasants, herons, and raptors including red kites) are known to have been victims of accidental or deliberate poisoning

by methiocarb (Giles *et al*., 1984; Greig-Smith *et al*., 1990; Fletcher *et al*., 1991; Fletcher *et al*., 1994). The recorded poisoning of hedgehogs by metaldehyde probably resulted from approved use of this material in slug pellets (M.R. Fletcher, pers. comm.). In this respect, it may be significant that slugs are known to form a large part of the diet of hedgehogs (Anon, 1964; Brockie, 1959). Thus, there is a real risk that hedgehogs, song thrushes (Baillie, 1993) and other wildlife species in which slugs form a substantial part of the diet, may succumb to secondary poisoning by molluscicides after feeding on slugs that have themselves eaten slug pellets. Equally, there is mounting evidence that some species (e.g. wood mice) are affected directly as a result of feeding on the pellets themselves (Johnson *et al*., 1992).

Although during the last two decades some non-chemical control methods have been tested in the agricultural and horticultural field (i.e. biological, cultural and varietal control methods), either their efficacy does not seem promising so far, or they have been prohibitively expensive (Glen and Moens, 2002). Therefore, the above chemicals still remain as the major effective control of slugs in both horticulture and agriculture. This fact has to be balanced with the accumulating evidence for their probable negative environmental impact. Furthermore, in the near future it is expected that baits will be constrained by tighter environmental protection constraints.

Slug damage to cereals (especially before germination) is particularly difficult to prevent and despite the widespread use of molluscicide baits (Glen and Wiltshire, 1992) it is probable that no more than 10% of a slug population can be destroyed using such methods (Fromming and Plate, 1952). In part, this is because baits have to maintain their comparative attractiveness against a variety of natural foods, despite the fact that the active ingredients may be repellent and may markedly reduce the amount of bait consumed (Wright and Williams, 1980; Wedgewood and Bailey, 1988). For these and other reasons, Henderson *et al*. (1992) concluded that there was little hope of improving the performance of existing slug baits.

As a consequence of the economic importance of slugs as pests, in the UK there is a large market for slug control products, and the increasing pest status of slugs is reflected by the sharp increase in molluscicide usage in recent years (Martin, 1991). In 1988 £8.9 million was spent on molluscicides, and 133 tonnes were applied to cereal crops. By 1990 this figure had increased to 309 tonnes of molluscicides which were used to treat 161,594 hectares of cereals - representing about 5% of the total area of cereals grown (Davis *et al*., 1991; Martin, 1991). Furthermore, molluscicide usage is also widespread among commercial horticulturists and private gardeners.

Thus, the discovery of novel, effective and environmentally safe techniques for slug control in agricultural and horticultural crops is urgently needed. Within this context it has been suggested that knowledge leading to the development of novel methods for the control of other invertebrate pests (for example endocrine disruption in insects) could also be applied to molluscs. Moreover, since molluscs have certain essential biochemical and behavioural processes that set them apart from other organisms, these could be exploited for control.

B. Establishment of a specific protocol for breeding terrestrial slugs under constant laboratory conditions

A specific protocol for breeding specific terrestrial slug species (in particular *D. reticulatum*, *A. subfuscus*, *Limax flavus*) under constant laboratory conditions has been established. Specifically for the grey field slug, *D. reticulatum* (which was the main pest slug of interest) it was shown that, in average, it reached the reproductively mature status 10-14 weeks after hatching, and that new hatclings emerged approximately 3 to 5 weeks after the eggs were laid. *D. reticulatum* was successfully cultured under certain abiotic (i.e. temperature, photoperiod, humidity) and biotic (i.e. density, artificial diet) conditions up to the F_3 generation (fig. 1). Thereafter, we enriched the breeding unit with slugs freshly collected from the field in order to avoid any inbreeding problems (within the colony) due to the relatively low number of slugs produced in the breeding unit.

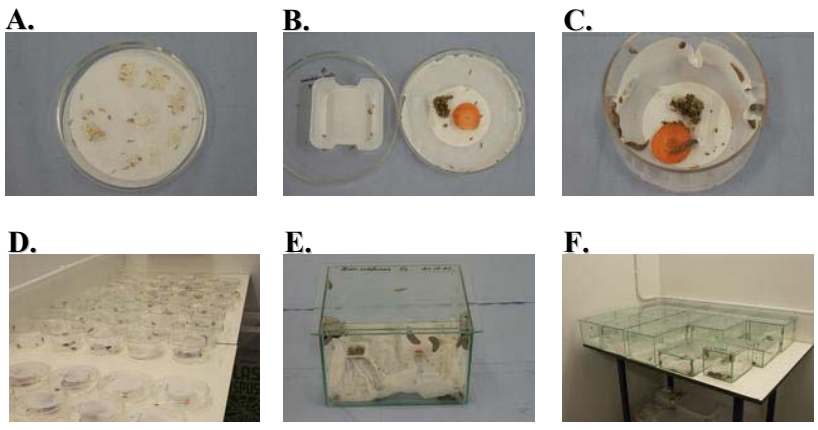

Figure 1: Details of the breeding unit of the grey field slug *Deroceras reticulatum* in CSL. A: newly hatched slugs; B & C: growth boxes of juvenile slugs; D & F: overview of the culture; E: reproductive box of adult slugs.

The establishment and the maintenance of this breeding colony of the above pest slug species has been considered as an essential initial step for the project because it allowed us:

i) to know the exact age of every specimen we use in either *in vitro* or *in vivo* bioassays, and subsequently

ii) to estimate the approximate physiological state of our specimens, and therefore reduce the biological variability that is commonly problematical studies on terrestrial pulmonate gastropods (i.e. terrestrial snails and slugs).

The importance of using reliable biological material, which would incorporate low level of uncertainty, for standardised tests with slugs with the view to test new molecules or develop new slug control

formulations has also been highlighted by other researchers (Christensen *et al*., 2003). Additionally, by establishing this breeding unit we ensured that we would have access to reliable biological material continuously throughout the year, without being dependent on the variable weather conditions and/or the abundance of slugs in the field.

C. Literature review of the current state of knowledge on the reproductive endocrinology of terrestrial slugs and snails

A comprehensive review of the current state of knowledge regarding the reproductive endocrinology of terrestrial pulmonate gastropods (i.e. terrestrial slugs and snails) has been completed during the first year of the project (the review was submitted to HGCA by the end of January 2001). In this review we have identified and described in detail the several endocrine and neuroendocrine centres that control reproduction in terrestrial pulmonate gastropods by triggering their respective target organs. Part of the above review has been published in the "Invertebrate Reproduction and Development" journal (2003, 44(2-3): 139-161).

The review emphasised the fact that most of the research on the reproductive physiology of terrestrial snails and slugs is now somewhat dated, and that many of the questions that emerged during the 70s, 80s and early 90s remain unanswered still. It also highlighted the continuing general lack of knowledge about the reproductive physiology of terrestrial pulmonate gastropods, by comparison with our understanding of these processes in freshwater representatives of this subclass. It was concluded that more research into the physiology of terrestrial pulmonate gastropods is needed in order to understand these processes in molluscs as a group, and it raised the possibility that the gastropod endocrine system could be targeted in order to develop precise, species-specific, more environmentally friendly control methods against terrestrial gastropod pests.

After evaluating all the information included in the review, the following endocrine organs were chosen as primary targets for further investigation and exploitation:

i) **The Dorsal Bodies Cells (DBCs)**: They consist of non-nervous endocrine cell bodies that are physically close to the central nervous system. In all pulmonate gastropods studied so far DBCs have been documented as one of the main endocrine centres involved in the control of reproductive activity. In particular, they are thought to control the maturation and synthetic activity of female associated parts of the reproductive system by secreting at least one gonadotrophic hormone (see literature reviews in Joosse, 1988; South, 1992; Saleuddin *et al*., 1994; Flari and Edwards, 2003), and they are involved in the control of oogenesis, oocyte maturation, differentiation and synthetic activity of accessory female sex organs and protein synthesis in the gonad (for more information see Flari and Edwards, 2003) (fig. 2). Nevertheless, the chemical nature of Dorsal Bodies Hormone(s), i.e. a steroid or a peptide/protein, is still under debate, and so far no Dorsal Body Hormone has been identified or characterised from any terrestrial or freshwater pulmonate gastropod.

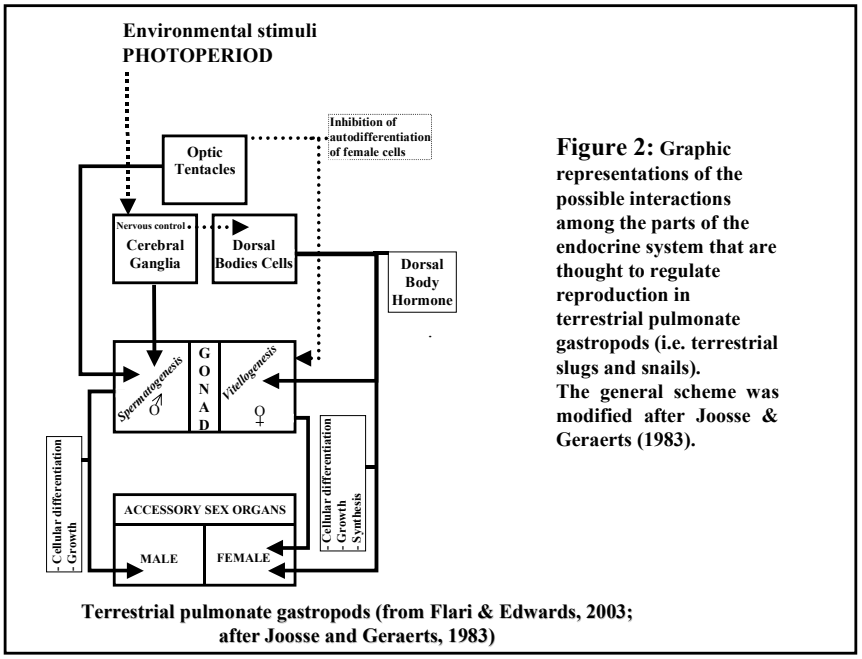

Figure 2: **Graphic representations of the possible interactions among the parts of the endocrine system that are thought to regulate reproduction in terrestrial pulmonate gastropods (i.e. terrestrial slugs and snails). The general scheme was modified after Joosse & Geraerts (1983).**

ii) **The Optic Tentacles**: Data regarding their possible endocrinological role are controversial (for detailed information see Flari and Edwards, 2003). In addition, it has been suggested that the optic tentacles of terrestrial pulmonate gastropods may participate in the physiological process of male maturation and in particular the one of protandry (a process that may take place in an hermaphroditic organism and during which the male reproductive system matures prior to the female one) that is commonly present in terrestrial slugs (fig. 2). Therefore, it could be considered as a potential target for endocrine disruption. However, we did not pursue any further research on the optic tentacles due to limited time and resources.

D. Investigation of the chemical nature of the main female hormone, the Dorsal Body Hormone (DBH), in terrestrial slugs and snails.

D. 1. Occurrence of ecdysteroids in the central nervous system of terrestrial pulmonate molluscs

Although the endocrine role of the Dorsal Bodies Cells has been known for some time, the precise chemical nature of the Dorsal Body Hormone(s) remains an unresolved issue (for more information see Flari and Edwards, 2003). In brief, some evidence suggests that these hormones may be proteinaceous (e.g. changes in the activity of Golgi zones, presence of elementary granules, variations in the exocytosis frequency in relation to changes in the endocrine activity of the Dorsal Bodies Cells). By contrast, other evidence indicates a steroid (specifically an ecdysteroid).

Our initial approach towards the question of the exact chemical nature of DBH was to test the hypothesis that DBH is an ecdysteroid, by investigating the presence of ecdysteroids in the central nervous

system, including the Dorsal Bodies Cells (DBCs), of terrestrial slugs and snails. The techniques employed for this investigation have been established in Central Science Laboratory previously, and they have been used for monitoring ecdysteroids in insects.

D. 1. 1. Methods and Materials

D. 1. 1. a. Biological material

Adult specimens of the terrestrial slug *D. reticulatum* were obtained from the breeding unit in CSL, whereas adult specimens of the terrestrial snail *Helix aspersa* were obtained from the field. Tissues of interest, in particular the cerebral ganglia and the Dorsal Bodies Cells, were excised into ice-cold physiological saline and kept at -20^{o}C until used.

D. 1. 1. b. Sample preparation

The samples from *D. reticulatum* were extracted for ecdysteroids according to methods described previously (Rees and Isaac, 1985; Magee *et al*., 1986; Mercer *et al*., 1987; Young *et al*., 1991). The samples from *H. aspersa* were extracted for ecdysteroids and subsequently fractionated by Reverse Phase High Performance Liquid Chromatography (RP-HPLC).

D. 1. 1. c. High-performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) of tissue extracts for ecdysteroid analysis was carried out using a Beckman SystemGold HPLC consisting of a programmable solvent module 126 and diode array detector 168 (Beckman Instruments (U.K.) Ltd.). Reversed-phase HPLC (RP-HPLC) was performed on a Waters Spherisorb S5ODS2 (25 cm x 4.6 mm i.d.; 250 Å) equilibrated with 30% methanol. Samples were loaded in 100 µl 30% aq. methanol and eluted over 40 min at 1 ml/min with a linear gradient of methanol to 100%. Fractions were collected each minute, dried under vacuum and stored at -20ºC until used.

D. 1. 1. d. Radioimmunoassay

The concentration of ecdysteroids in the samples extracted for ecdysteroids (in the slug *D. reticulatum*) and those that were fractionated in RP-HPLC (in the snail *H. aspersa*) was determined by radioimmunoassay (RIA) according to the method described previously (Borst and O'Connor, 1972) with minor modifications. The polyclonal antiserum used in this study was produced by Dr. W.E. Bollenbacher (Univ. North Carolina, Chapel Hill) and distributed by Dr. E. S. Chang (Bodega Marine Laboratory, Bodega Bay, CA). This antiserum was generated against a 2-succinyl derivative of ecdysone and has a high affinity for both ecdysone and 20-hydroxyecdysone in the ratio of approximately 2:1. The unlabelled ligand used in generating the standard curve was 20-hydroxyecdysone, and thus ecdysteroid measurements are expressed in 20-hydroxyecdysone equivalents.

D. 1. 2. Results and Discussion

In the experiments performed we have been unable to detect any immunoreactive ecdysteroids in any of the extracts of the tissues tested, either the cerebral ganglia or the Dorsal Bodies Cells, of either the slugs (i.e. *D. reticulatum*) or the snail species (*H. aspersa*) that we have tested. Furthermore, the RP-HPLC profiles

of the tissue extracts that we obtained (fig. 3: solid pale line) showed that there is no peak that co-migrates with the standards of 20-hydroxy-ecdysone or ecdysone from either the Dorsal Body Cells or the Cerebral Ganglia extracts (fig. 3: dotted pale line).

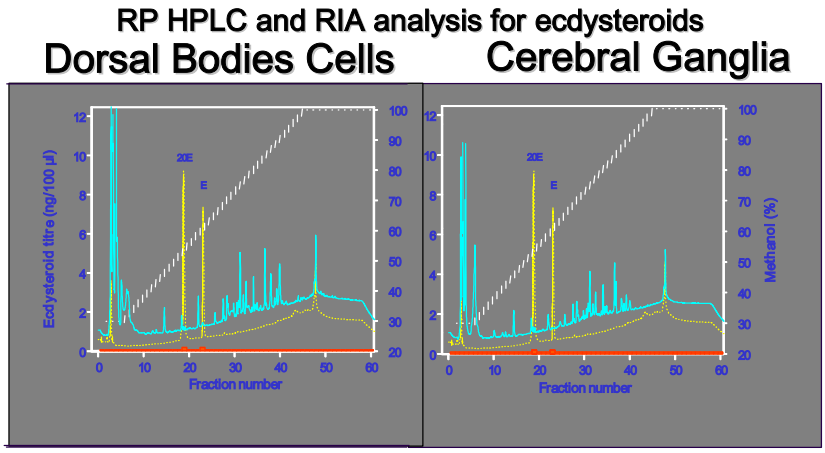

Figure 3: **Reverse phase HPLC and RIA analysis of ecdysteroids in extracts from Dorsal Bodies Cells and from the neuronal elements of cerebral ganglia of the terrestrial snail Helix aspersa. Blue line: RP-HPLC chromatograms of the tissues' extracts; yellow line: chromatography of 20-hydroxy-ecdysone and ecdysone standards; red line: RIA analysis for ecdysteroids of the fractionated tissues' extracts.**

In addition, we performed RIA (for immunoreactive ecdysteroids) on all fractions from RP-HPLC of either the cerebral ganglia or the Dorsal Bodies Cells. Unsurprisingly, we were unable to detect any immunoreactive ecdysteroids (fig. 3: solid dark line) in any of the fractions produced by RP-HPLC, therefore providing further evidence against the hypothesis that an ecdysteroid is present in the cerebral ganglia or the endocrine Dorsal Body Cells in terrestrial slugs and snails.

The animals used in the above experimental trials were reproductively mature and in particular in the hermaphroditic stage, as indicated by the Maturation Index of their albumen gland [Maturation Index = ($Weight_{AG}$/Body weight)*100: Bride and Gomot, 1995]. In terrestrial pulmonates, van Minnen and Sokolove (1984) first reported the synchronisation between the time at which the DBCs reached their largest size (and presumably, their highest synthetic activity), and the female reproductive phase in the slug *L. maximus*. Ezzughayyar and Wattez (1989) reported similar correlations in another slug, *A. rufus*, where the DBCs were inactive during the infantile and male phases, but reached their maximal activity during a) the late juvenile phase (corresponding to the phase of oocyte growth and the peak of galactogen synthesis in the albumen gland), and b) the female phase, particularly at the time of egg laying (i.e. corresponding to the maximal development of FASO). Considering all the above, it appears that the likelihood that the Dorsal Body Hormone is an ecdysteroid in the terrestrial slug and snail species that we were interested in is minimal.

Our results are in contrast though with studies supporting the presence of ecdysteroids in the Dorsal Bodies Cells (Krusch *et al*., 1979; Nolte *et al*., 1986; Mukai *et al*., 2001) as well as with these that indicate a

biological functional role for ecdysteroids in pulmonate molluscs including freshwater and terrestrial snails (Bride *et al*., 1991; Shiff and Dossaji, 1991). The debate concerning the possible steroidal nature of the Dorsal Body Hormone, has involved questions relating to the ability of pulmonates to accomplish the biochemical synthesis of ecdysteroids and other steroid molecules. The debate as regards the presence of ecdysteroids as well as the capability of pulmonate molluscs to synthesise them *in vivo* was fuelled by Garcia and her colleagues (Garcia *et al*., 1995). In this study, several terrestrial pulmonates, including the snails *H. aspersa*, *Cepaea nemoralis*, *C. hortensis*, and *Arianta arbustorum*, and the slug *D. reticulatum*, were tested for the presence of the enzymes required for the conversion of certain precursors (e.g. 2-deoxyecdysone, 3-dehydro-5β-2,22,25-trideoxy-ecdysone, cholesterol, etc.) into ecdysone. Although some of the enzymes necessary for the conversion of some precursors (e.g. 3-oxoecdysteroid 3β-reductase) were detected, no 2-ecdysone hydroxylase or 22-ecdysone hydroxylase activity (necessary in arthropods for the complete conversion of cholesterol to ecdysone) could be detected. On the basis of these results, the authors concluded that, if terrestrial pulmonates do in fact synthesise ecdysteroids, then they might use a different biosynthetic pathway to that used by insects. It is worth remembering that insects cannot synthesize steroids *de novo*, and that all insect steroids are derived from primary (dietary) steroid molecules such as cholesterol and β-sitosterol. However, there is evidence (le Guellec *et al*. 1987; Wootton *et al*. 1995) indicating that some molluscs are able to synthesize vertebrate-like steroids (e.g. testosterone) from simple precursors, and such molecules have been shown to be present in several slug and snail species.

The inability to prove the presence of any ecdysteroids in either the cerebral ganglia or the Dorsal Bodies Cells in our experiments supports the hypothesis that the Dorsal Body Hormone is a protein. Ebberink *et al*. (1983) were the first to describe a protein, with a MW of about 30kDa, which they partially purified from the DBCs of the aquatic snail *L. stagnalis*. Interestingly, Ebberink *et al*. (1983) mentioned that DBH appeared to lose at least some of its biological activity (specifically the ovulation inducing activity), upon partial purification. The possibility that DBH is proteinaceous was strengthened by data showing that the putative hormone was pronase sensitive (Wijdenes *et al*. 1983; de Jong-Brink *et al*. 1986), and that it activated adenylate cyclase-cAMP in *in vitro* gonad cultures (de Jong-Brink *et al*. 1986). Saleuddin *et al.* (1989) demonstrated that DBCs in *H. duryi* are capable of rapid incorporation of leucine - as might be expected to occur in a typical protein-synthesising cell. Finally, Teunissen *et al*. (1992) reported the isolation of a cytochrome P_{450} sequence in cDNA clones from the DBCs of the aquatic snail *L. stagnalis*. This cytrochrome P_{450} was a representative of a separate family of these proteins, and the mRNA was uniquely and abundantly expressed in the DBCs. Teunissen *et al*. (1992) argued that the presence of only one cytochrome P_{450} in the DBCs was indicative of a probable proteinaceous DBH, since steroid synthesis usually requires several cytochrome P_{450} enzymes belonging to different families. However, these authors also indicated that the sequence of the *Lymnaea* enzyme showed the highest similarity with mitochondrial cytochrome P_{450} enzymes known to be involved in the synthesis of steroid hormones.

In the case of terrestrial pulmonates, there has also been some (albeit indirect) evidence that DBH may be a protein. Goudsmit and Ram (1982) and van Minnen and Sokolove (1984) showed that the DBCs are the major source of a galactogen synthesis stimulating factor (Gal-SF) in the snail *H. pomatia* and the slug *L. maximus*, respectively. Gel filtration chromatography indicated that this Gal-SF (which may be identical to DBH) was probably a protein with a molecular weight of between 3 and10 KDa. However, in these studies the DBCs used were not separated from the underlying cerebral ganglia, and therefore the preparation and purification of the Gal-SF was made from whole brain homogenates (i.e. not solely from isolated DBCs). In the freshwater snail *H. duryi*, Miksys and Saleuddin (1988) reported that there were two factors, present in whole brain homogenates, both of which stimulated polysaccharide synthesis in *in vitro* cultures of albumen glands. They suggested that one of these factors might be the DBH from the DBCs, and the other, a factor from the neuronal element of the cerebral ganglia, which might be an egg-laying hormone, and which may be similar or identical to the caudo-dorsal cell hormone (CDCH) that induces egg-laying in freshwater pulmonates (Geraerts & Joosse, 1984; Ebberink *et al*. 1985). Although, in terrestrial pulmonates, initial studies indicated that an egg-laying hormone possibly originated from cells in the parietal ganglia (Griffond *et al*. 1992), the possibility that more than one polysaccharide synthesis stimulating factor may exist in whole brain homogenates can not be ruled out.

Considering all the above it is clear that further studies on the steroidogenesis as well as on the chemical nature of this important female reproductive hormone, the Dorsal Body Hormone, in terrestrial slugs and snails are warranted.

D. 2. The use of Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI/TOF-MS) as a potential tool for investigating peptidic profiles directly in cells and/or tissues in terrestrial slugs and snails

Following the results indicating the absence of any ecdysteroids in the central nervous system of terrestrial slugs and snails our research was directed towards the investigation of the putative peptidic or proteinaceous nature of Dorsal Body Hormone. To this end, we have developed a technique based on Matrix Assisted Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (MALDI/TOF-MS) in order to investigate and characterise peptides and proteins associated with the (neuro)endocrine centres in the central nervous system in terrestrial slugs and snails.

During the last two decades the technique of MALDI/TOF-MS has proven to be a fast, reliable and sensitive technique to detect the endogenous peptides and their patterns in complex biological systems. The advantages of this technique include its high speed (seconds) and its high sensitivity (femtomole range). Direct MALDI/TOF-MS was also proven to be an excellent tool for detecting (and possibly semi-quantifying) profiles of peptides in tissues and single cells (Dreisewerd *et al*., 1997; Jimenez and Burlingame, 1998; Page *et al*., 2000). The use of mass spectrometry for the analysis of cellular peptide profiles present in neuroendocrine organs is based on the rationale that measurement of the molecular weight of these peptides is sufficiently specific in order to identify peptides for which it has been known that they are present in the

tissue that is tested. More importantly, screening of (neuro)endocrinological tissues by MALDI/TOF-MS may provide essential qualitative information as regards the peptidic profiles at different physiological stages of the animals. Furthermore, it may be combined with further biochemical and molecular biology techniques (such as HPLC, cDNA technology, etc.) in order to identify and characterise further the peptides of interest.

Experimental trials that involved screening of the central nervous system tissues with direct MALDI/TOR-MS were conducted during the 2nd year of the research project, and they were restricted only to "proof-of-concept" experiments due to limited resources.

D. 2. 1. Methods and Materials

D. 2. 1. a. Biological material

A. subfuscus and *D. reticulatum* specimens used were reared in our laboratory under controlled environmental conditions (15°C, L:D 14:10, R.H. 95%), and they were of known age and physiological status. *H. aspersa* and *L. maximus* specimens were collected from the field and kept under constant laboratory conditions (15°C, L:D 14:10, R.H. 95%) until used.

D. 2. 1. b. Preparation of samples

The tissues of interest, in particular the connective sheath surrounding the cerebral ganglia, were removed by micro dissection under a stereomicroscope. Consequently, the samples for insertion into the mass spectrometer were prepared according to two different strategies / approaches. In the first approach, we transferred the isolated tissue by tungsten needles to a stainless-steel MALDI target plate containing 0.5μl of aqueous 2,5-dihydroxybenzoic acid (DHB: 10mg/ml). The tissue was then transferred successively to a fresh matrix drop on a different spot on the MALDI target plate. Such spot-to-spot transfers were repeated for a number of times (3-5). In the second approach, the physiological saline used during the micro dissection was substituted by the matrix solution [in this case 2,5 DHB (10mg/ml)] so that the salt removal would occur simultaneously with the tissue dissection. Tissue was then placed on a fresh 0.5μl drop of aqueous 2,5 DHB (10mg/ml) on a spot on the MALDI target plate, and the plate was subsequently inserted into the mass spectrometer for acquisition of data.

D. 2. 1. c. Instrumentation

MALDI-TOF-MS was performed on a Voyager DE-STR (Applied Biosystems, Warrington, UK). The instrument was operated in positive linear mode up to a mass of 50,000. The accelerating voltage was 25000 V with an extraction delay time of 400 nsec. Data acquisition software was Voyager Control Panel 5.1 with Data Explorer 4.0 processing software.

D. 2. 2. Results and Discussion

The results obtained revealed that, irrespective of the terrestrial slug or snail species studied, a large number of peptides and proteins (with a molecular weight between 1000 Da and 10,000 Da) are present in the connective sheath that surrounds the cerebral ganglia and includes the Dorsal Bodies Cells in terrestrial pulmonate gastropods (fig. 4). Additionally, we showed that direct MALDI/TOF-MS on the tissue of Dorsal

Bodies Cells produced consistently better results as compared with MALDI/TOF-MS performed on homogenised and partially cleaned samples.

Furthermore, despite of the limited experimental trials that we finally performed, we were able to demonstrate that the specific experimental protocol which we have developed can produce reliable results (fig. 4).

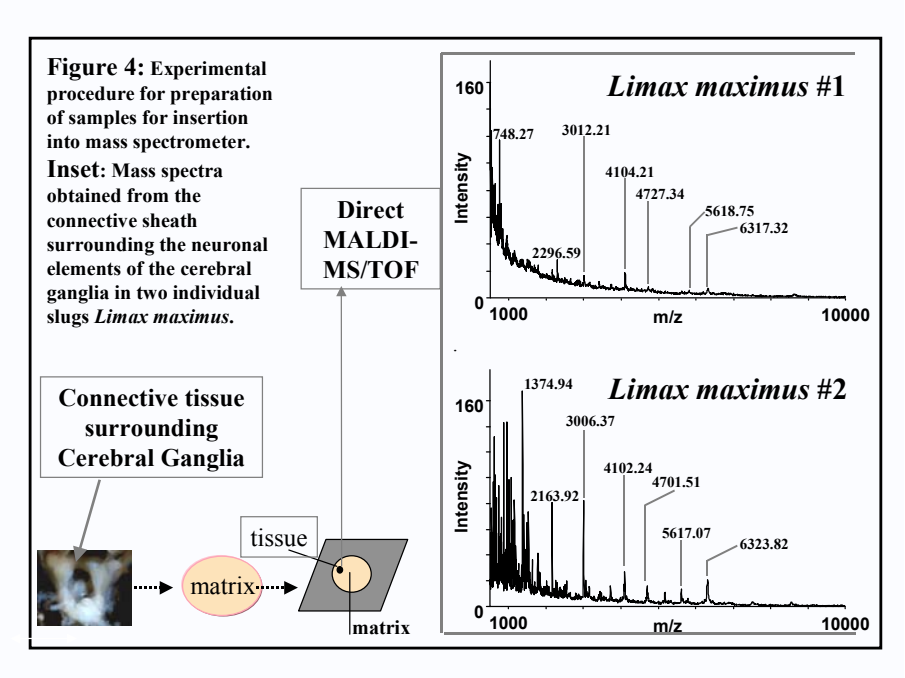

Figure 4: **Experimental procedure for preparation of samples for insertion into mass spectrometer.** **Inset: Mass spectra obtained from the connective sheath surrounding the neuronal elements of the cerebral ganglia in two individual slugs *Limax maximus*.**

D. 3. Establishment of an *in vitro* bioassay with the view to a) screen for biological activity of Dorsal Body Hormone (DBH), b) investigate the inhibition of DBH function, and c) evaluate potential endocrine-disruptive molecules of the female reproductive maturation in terrestrial pulmonate molluscs

The Dorsal Bodies Cells in pulmonate molluscs are involved in second-order and possibly in third-order (neuro)endocrine cascades that control the maturation and synthetic activity of female associated parts of the reproductive system in pulmonate molluscs, including freshwater and terrestrial representatives. The albumen gland is one of the female sex accessory organs in these animals, and its main function is the secretion of the perivitalline fluid, which is rich in polysaccharides and in particular galactogen, that surrounds each fertilised egg. So far, it has been well documented that the maturity and secretory activity of the albumen gland is directly controlled by the female gonadotropic hormone(s), one of which is the Dorsal Body Hormone (Goudsmit and Ram, 1982; van Minnen *et al*., 1983; van Minnen and Sokolove, 1984; Miksys and Saleuddin, 1987; Mukai *et al*., 2001).

During the 2nd year of our research project we decided that the establishment of a reliable bioassay -that can detect Dorsal Body Hormone biological activity by measuring the ^{14}C incorporation in polysaccharide

synthesis in *in vitro* culture of albumen gland- was essential in order to be able to characterise further the main female hormone in terrestrial slugs and snails.

D. 3. 1. Methods and Materials

D. 3. 1. a. Biological material

Specimens of *D. reticulatum*, *L. maximus* and *A. subfuscus* were obtained from our slug-breeding unit. All the animals that were used as "donors of albumen glands" were starved for 24hours before their albumen gland was removed. After the animals had been weighed, the albumen gland was removed and the maturation index (MI: as indicated above) of the slugs was calculated.

D. 3. 1.b. Preparation of albumen gland explants

The excised albumen glands were separated by any remnants of hermaphroditic gland tissue, rinsed in physiological saline solution and subsequently they were sterilised by passing them through a successive series of baths (5min each) in petri dishes containing sterile physiological saline to which decreasing quantities of penicillin-streptomycin were added (1000, 500 and 100 U).

D. 3. 1. c. Preparation of culture medium

A culture medium modified from that described previously (van Minnen *et al*., 1983) was introduced into sterile COSTAR culture plates with 12 chambers. Into each chamber there was pipetted 900ml of appropriated medium to which 1.5μCi and 150 U of penicillin-streptomycin was added before introducing the albumen gland explants.

D. 3. 1. d. Preparation of cerebral ganglia and/or Dorsal bodies extracts

Extracts of "cerebral ganglia + dorsal bodies cells complexes" (Ce-DBCs complexes) were prepared by homogenizing in the appropriated medium on ice, twice and centrifuging at 13,200*g* for 10min at 4^{o}C.

D. 3. 1. e. Experimental procedure for determining ^{14}C incorporation

At the end of the culture period (up to 48 hours) the weight of the albumen glands explants was calculated. Consequently, they were homogenised in 10% TCA and centrifuged 13,200*g* for 10min at 4^{o}C. Polysaccharide synthesis in albumen glands was determined in the supernatants by measuring the incorporation of ^{14}C according to the methods described previously (van Minnen *et al*., 1983; Miksys and Saleuddin, 1988; Mukai *et al*., 2001).

D. 3. 1. f. Statistical analysis of data

Data (dpm/mg wet weight of albumen gland explants) were statistically analysed by ANOVA after normalisation with log transformation. Paired comparisons between the different experimental groups were performed with Fisher's test.

D. 3. 2. Results and Discussion

We have successfully established a bioassay that can be used to estimate the Dorsal Body Hormone biological activity *in vitro*. In essence, the bioassay determines the incorporation of ^{14}C in polysaccharide synthesis in *in vitro* culture of the albumen gland explants.

The results acquired demonstrate that explants of the albumen gland of terrestrial slugs, in particular the species *D. reticulatum* and *A. subfuscus*, can be cultured long term (up to 48hs) *in vitro*. The results obtained indicate that polysaccharide biosynthesis in the albumen gland of terrestrial slugs can be monitored by measuring the ^{14}C-glucose incorporation into polysaccharides that are naturally synthesised in the albumen gland of these animals (mainly galactogen and glycogen). The *in vitro* experiments performed showed that polysaccharide biosynthesis is stimulated in the presence of extracts from the cerebral ganglia with the Dorsal Bodies Cells attached (namely "cerebral ganglia + dorsal bodies cells complexes": "CG+DBCs complexes") (figs. 5-10). Nevertheless, the actual increase in polysaccharide synthesis varied among the several experimental trials, with values ranging from 2-fold to 5-fold increase in individual albumen gland explants. Additionally, the absolute amount of stimulation of polysaccharide synthesis varied a lot, ranging from approximately 400 to 5,000 dpm/mg wet weight$_{AG}$ (figs. 5-10).

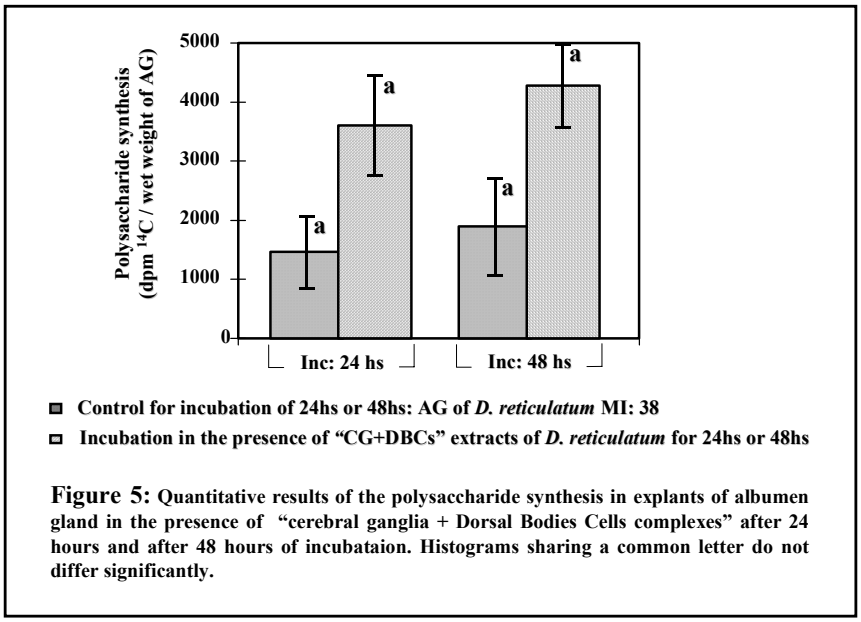

Figure 5: Quantitative results of the polysaccharide synthesis in explants of albumen gland in the presence of "cerebral ganglia + Dorsal Bodies Cells complexes" after 24 hours and after 48 hours of incubataion. Histograms sharing a common letter do not differ significantly.

This variability has been shown in previous studies (van Minnen *et al*., 1983; van Minnen and Sokolove, 1984), and possible reasons for this include the difference of the size of the albumen gland explants as well as the differences in the actual physiological state of the albumen glands. Nevertheless, it is important to note that, despite of the recorded differences among the several experimental trials, the stimulation of polysaccharide biosynthesis by the "CG+DBCs complexes" was consistent.

In order to determine the optimum time of incubation of the albumen gland explants, with the aim to attain the highest rate of stimulation of the polysaccharide synthesis in the presence of extracts from "cerebral ganglia + Dorsal Bodies Cells complexes", we sampled our bioassay after 24 and 48 hours of incubation (fig. 5). It was shown that the rate of stimulation of polysaccharide synthesis that was achieved after 48 hours of incubation was similar to that achieved after 24 hours. Consequently, the duration of 24 hours was chosen as the optimum time of incubation for further experiments (fig. 5).

In order to study whether the secretion of these polysaccharide synthesis stimulating factors (POL-SFs) changes in relation to the female maturity phase of the slugs, we tested the effects of "CG+DBCs complexes" originating from slugs that were at different maturation phases as indicated by the exact Maturation Index of their albumen gland (figs. 6,7)

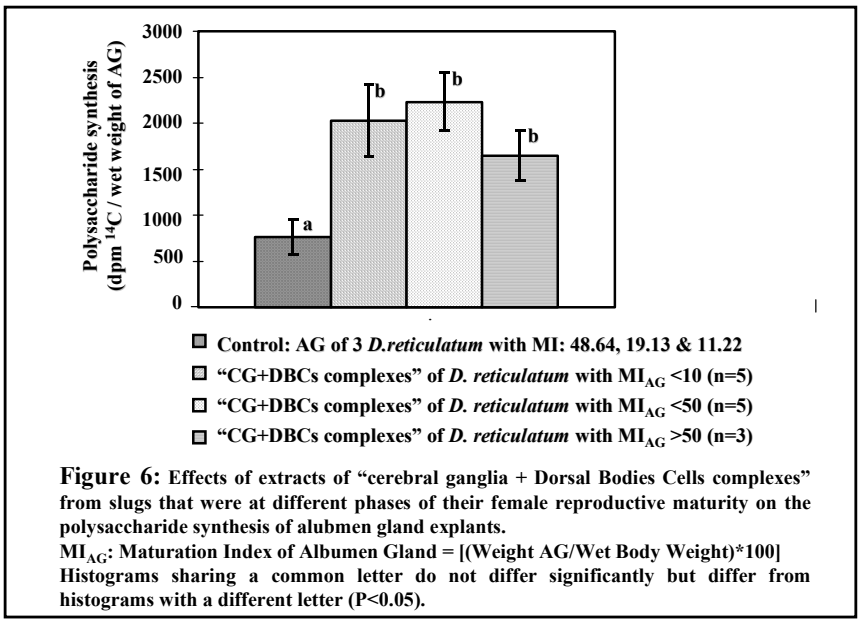

Figure 6: Effects of extracts of "cerebral ganglia + Dorsal Bodies Cells complexes" from slugs that were at different phases of their female reproductive maturity on the polysaccharide synthesis of alubmen gland explants.
MI$_{AG}$: Maturation Index of Albumen Gland = [(Weight AG/Wet Body Weight)*100]
Histograms sharing a common letter do not differ significantly but differ from histograms with a different letter (P<0.05).

It was shown that the POL-SFs were secreted in high quantities as soon as the animals enter their female phase of reproductive maturity, since the "CG+DBCs complexes" from slugs with MI$_{AG}$ <10 stimulated the polysaccharide synthesis in the albumen gland in a similar way to the slugs with higher MI$_{AG}$ (figs. 6-7). Thereafter, the secretion of the POL-SF remained at high levels (figs. 6-7), and appeared to decline when the animals entered the late phases of their reproductive activity and their MI$_{AG}$ were quite high (fig. 7).

In the freshwater snail *H. duryi*, Miksys and Saleuddin (1988) reported that there were two factors, present in whole brain homogenates, both of which stimulated polysaccharide synthesis in *in vitro* cultures of albumen glands. They suggested that one of these factors might be the Dorsal Body Hormone from the DBCs, and the other, a factor from the neuronal element of the cerebral ganglia, which might be an egg-laying hormone, and which may be similar or identical to the caudo-dorsal cell hormone (CDCH) that induces egg-laying in freshwater pulmonates (Geraerts and Joosse, 1984; Ebberink *et al*., 1985). Although, in terrestrial pulmonates, initial studies indicated that an egg-laying hormone possibly originated from cells in the parietal ganglia (Griffond *et al*., 1992), the possibility that more than one polysaccharide synthesis stimulating factor may exist in whole brain homogenates can not be ruled out. In order to test the possibility that there is more than one POL-SF in the central nervous system of the grey field slug, we tested the effects of the neural fraction of the cerebral ganglia and of the connective sheath that surrounds the cerebral ganglia (and includes the Dorsal Bodies Cells) on polysaccharide synthesis separately (fig. 8).

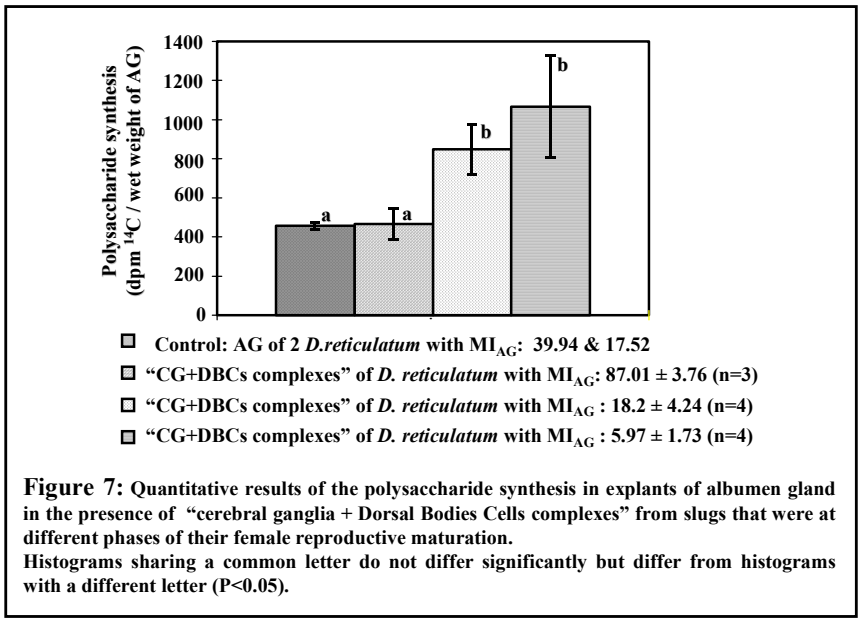

Figure 7: **Quantitative results of the polysaccharide synthesis in explants of albumen gland in the presence of "cerebral ganglia + Dorsal Bodies Cells complexes" from slugs that were at different phases of their female reproductive maturation.**
Histograms sharing a common letter do not differ significantly but differ from histograms with a different letter (P<0.05).

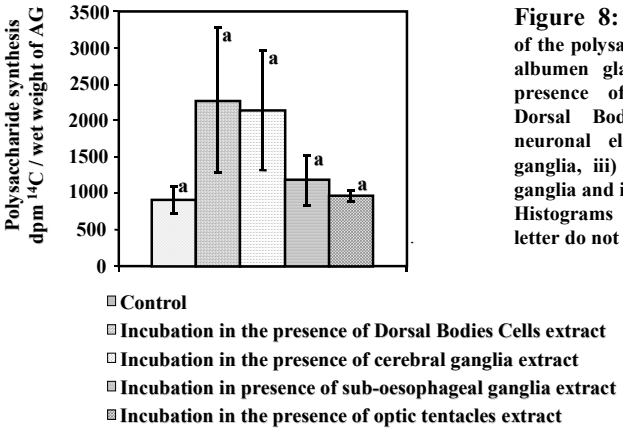

Figure 8: **Quantitative results of the polysaccharide synthesis in albumen gland explants in the presence of extracts from i) Dorsal Bodies Cells, ii) the neuronal elements of cerebral ganglia, iii) the sub-oesaphageal ganglia and iv) the optic tentacles. Histograms sharing a common letter do not differ significantly.**

The results obtained showed that the rate of the stimulation of polysaccharide synthesis by extracts from the Dorsal Bodies Cells and by these from the neural components of the cerebral ganglia were similar, although the actual increase in the polysaccharide synthesis provoked by these extracts did not differ statistically significantly from the controls (fig. 8). Interestingly, the sub-oesophageal ganglia (containing the parietal ganglia for which previous research indicated that they may be the source of the egg-laying hormone in terrestrial pulmonate gastropods) did not induce any increase in polysaccharide synthesis, perhaps because the animals used in these experimental trials were not involved in mating and/or egg-laying activities (fig. 8).

Goudsmit and Ram (1982) and van Minnen and Sokolove (1984) showed that the Dorsal Bodies Cells are the major source of a galactogen synthesis stimulating factor (Gal-SF) in the snail *H. pomatia* and the slug *L. maximus*. By using gel filtration chromatography they provided evidence that this Gal-SF (which may be identical to Dorsal Body Hormone) was probably a protein with a molecular weight of between 3,000 and10,000 Da. We fractionated "CG+DBCs complexes" into small molecules, including peptides and proteins smaller than 5,000 Da, and large molecules, including proteins larger than 5,000 Da, by using cut-off membranes (at 5,000 Da), and then we tested those fractions individually (fig. 9). The results obtained indicated that polysaccharide synthesis in the albumen gland explants was stimulated significantly by both fractions (fig. 9), indicating that there may be more than one polysaccharide stimulating factor in the central nervous system of terrestrial slugs.

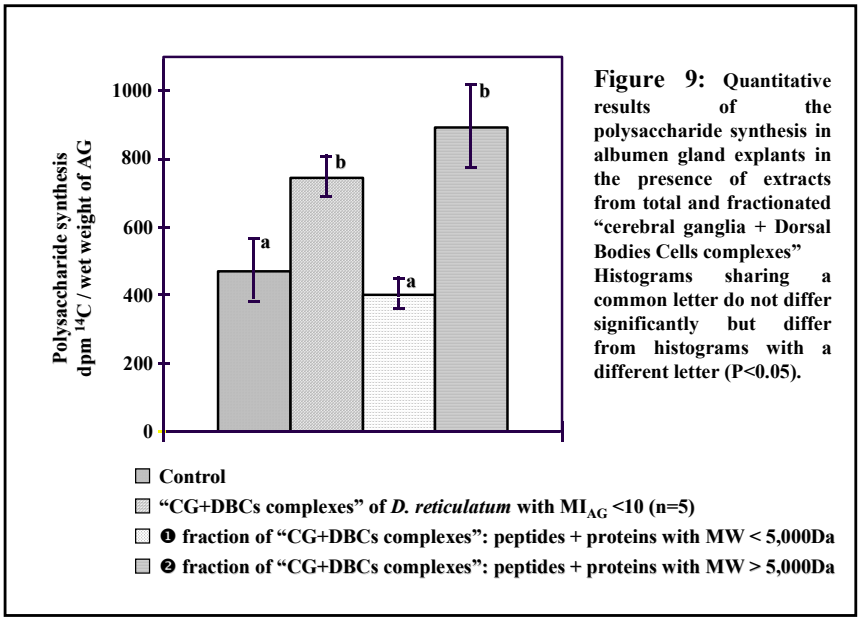

Figure 9: Quantitative results of the polysaccharide synthesis in albumen gland explants in the presence of extracts from total and fractionated "cerebral ganglia + Dorsal Bodies Cells complexes" Histograms sharing a common letter do not differ significantly but differ from histograms with a different letter (P<0.05).

Attia *et al*. (1998) provided some interesting results that indicated a possible temporal pattern of the activity in the cerebral green cells (Ce-GCs) of the terrestrial snail *H. aspersa*. It appears that the synthesis of neurosecretory materials in Ce-GCs varies daily, showing a peak at the onset of activity of the animals. In contrast, the secretory activity of the Dorsal Bodies Cells occurs primarily during the inactive phase of the animals (Mounzih *et al*. 1988). The above observations support the hypothesis that Cerebral Green Cells exert an inhibitory control on DBCs. Additionally, these data provide further evidence for the probable involvement of endogenous timing mechanisms (e.g. annual or circadian rhythms) in the reproductive endocrinology of pulmonate gastropods.

In order to assess whether the Dorsal Bodies Cells activity show a temporal pattern in their activity we tested polysaccharide synthesis stimulation by "CG+DBCs complexes" in the slug *A. subfuscus* at different times during a 24 hour photoperiod cycle in the laboratory (L:D 14:10). It was shown that the polysaccharide synthesis stimulating factors (POL-SFs) were present in the central nervous system of the slugs continuously,

during the 24 hours photoperiod cycle (fig. 10A). However, the "CG+DBCs complexes" that were removed from the slugs during the light hours appeared to stimulate the polysaccharide synthesis in the albumen gland explants more (albeit not statistically significantly; fig. 10A) as compared with these removed from the slugs during the dark hours of the photoperiod cycle. These results are in accordance with previous histological observations indicating that the secretory activity of the Dorsal Bodies Cells in terrestrial snails is mainly taking place during the inactive phase of the animals, that is during the light hours (Mounzih *et al*., 1998). We also attempted to detect the presence of the POL-SFs in the haemolymph of the slugs (fig. 10B). Nevertheless, the stimulation of polysaccharide synthesis in the albumen gland explants by the haemolymph extracts was not significantly higher than the controls, perhaps because the quantity of the respective hormones into the haemolymph is minute or the hormone(s) get metabolised very quickly after their release into the blood (fig. 10B).

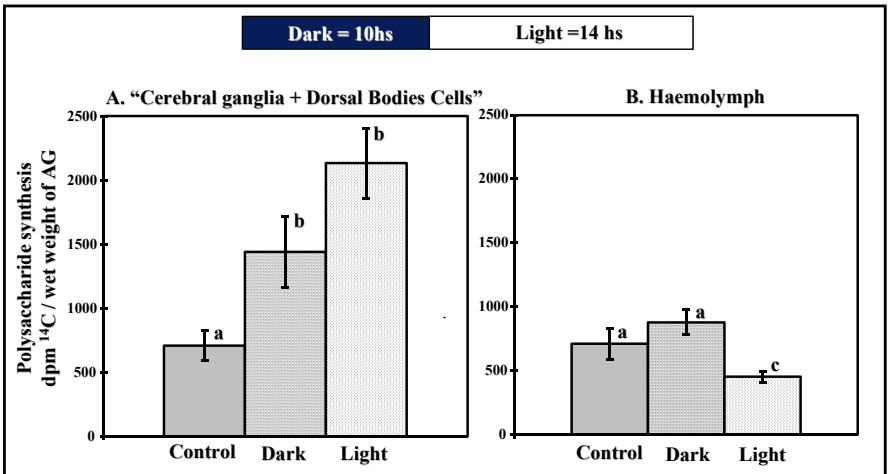

Figure 10: **Quantitative results of the polysaccharide synthesis in explants of albumen glands in the presence of "cerebral ganglai + Dorsal Bodies Cells complexes" (A) and the haemolymph (B) during the dark and the light hours of a 24 hour cycle under laboratory condtions (L:D 14:10). Histograms sharing a common letter do not differ significantly but differ from histograms with a different letter (P<0.05).**

E. Investigation of the potential of the lectin *Galanthus nivalis* agglutinin (GNA) as a carrier molecule for novel slug control systems and evaluation of *Manduca sexta* allatostatin (Manse-AS) as a possible feeding inhibitor in terrestrial slugs with the view to i) develop practical techniques for delivery of novel molluscicidal peptides by oral administration, and ii) assess the potential of the fusion protein of GNA-Manse-AS as a novel slug control method

Lectins from snowdrop (i.e. *Galanthus nivalis* agglutinin) are known to have toxic effects on several insect pests (e.g. *Nilaparvata lugens*, *Lacanobia oleracea*: Gatehouse *et al*., 1996, 1997). Insecticidal effects of GNA include reduced larval growth rate, as well as reduced food consumption and eventually reduced

survival of insects. Although, the exact mechanism by which GNA exerts its insecticidal activities is not known, it has been shown that when the lectin is fed to insects it can accumulate *in vivo* in the gut, the malpighian tubules and the haemolymph of the animals (Fitches *et al*., 2001).

Neuropeptides in invertebrates, including molluscs, are involved in the regulation of many aspects of their physiology, and can be the basis for effective and highly selective methods for pest control in the field. However, their potential as pest control agents is reduced because neuropeptides are likely to degrade rapidly in the environment, as well as in the digestive system of the animals. In our laboratory it has been shown that a fusion protein that combines an insect-neuropeptide, i.e. *Manduca sexta* allatostatin (Manse-AS), with snowdrop lectin (GNA) can successfully transport the peptide to the insect haemolymph following oral ingestion (Fitches *et al*., 2002). Furthermore, larvae exposed to the fusion protein diet cease to feed and eventually die (Fitches *et al*., 2002).

GNA effects on the physiology of terrestrial pulmonate gastropods have not been tested so far. Therefore, during the 2nd year of our research project we conducted some short-term experiments regarding any possible effects of GNA on food consumption and survival of the grey field slug *D. reticulatum*. The results obtained indicated that addition of GNA as a component of the artificial diet (2% w/w) delivered to slugs only slightly reduced the food consumption of adult slugs, and had no effect on their survival. Nevertheless, we tested whether snowdrop lectin could also be transported into the haemolymph of terrestrial gastropods, with the view to its eventual use as a carrier of neuropeptides that may inhibit certain physiological processes, such as development or reproduction. The current knowledge on such regulatory neuropeptides and/or (neuro)hormones in molluscs, particularly in the terrestrial gastropods, however, is relatively poor. Manse-AS is a known feeding and hormone biosynthesis inhibitor in insects (Weaver *et al*., 1998; Audsley *et al*., 2001) and allatostatin-like immunoreactivity in pulmonate gastropods (i.e. *L. pseudoflavus*) and freshwater molluscs (i.e. *Bulinus globosus*, *Stagnicola elodes*) has been reported previously (Halton and Buchanan, 1994; Rudolph and Stay, 1997). However, this immunoreactivity was associated with allatostatin peptides derived from the cockroach *Diploptera punctata*, and these "cockroach allatostatins" have no structural homology with the Manse-AS molecule. Nevertheless, we decided to investigate whether synthetic Manse-AS could act as a feeding inhibitor in slugs, in order to evaluate the potential of the GNA/Manse-AS fusion protein as a control agent of pest slugs in the field.

E. 1. Methods and Materials

E. 1. a. Biological material

A. subfuscus and *D. reticulatum* were used in experiments investigating the uptake of GNA and its subsequent appearance in the blood. The effects of Manse-AS *in vivo* were assayed only on the grey field slug *D. reticulatum*. All slugs used were reared in our laboratory under controlled environmental conditions (20^{o}C, L:D 14:10, R.H. 95%), and they were of known age and physiological status.

E. 1. b. Administration of GNA - sampling of tissues for detection of GNA – detection of GNA

Snowdrop lectin, supplied by Dr E. van Damme, Catholic University of Leuven, BELGIUM, was administered orally [at 2% (w/w) of total dietary protein] in the artificial diet presented to individual, isolated slugs. The feeding activity of the slugs was recorded daily. Those slugs that were noted to have consumed a substantial quantity of diet were anaesthetised by being placed on ice for 15-30min. Subsequently, the haemolymph from these animals was obtained, and the separate parts of the digestive system were dissected and removed. Faeces produced by these animals during the 24hours were also collected. The dissected parts and the faeces were extracted in 0.1M Tris buffer (pH 7.4) and centrifuged at 13,200*g* for 10min at 4^{o}C. The protein content of all samples was estimated by a microtitre plate-Bradford Assay developed by Biorad and using BSA as the standard protein. The presence of GNA in these samples was determined with western blot analysis as described previously (Fitches *et al*., 2001).

E. 1. c. Synthesis of *Manduca sexta* allatostatins (Manse-AS)

Manduca sexta allatostatin was custom synthetised using solid phase methodology (Fmoc procedure) on an Applied Biosystems model 431A automatic peptide synthesiser at the Advanced Biotechnology Center, Imperial College School of Medicine at Charing Cross Hospital, London, UK.

E. 1. d. Experimental procedure for injecting synthetic Manse-AS

Synthetic Manse-AS was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 5μg/μL (because of its relative insolubility in water). Manse-AS was injected in a ratio of 5μg/100mg of wet body weight in order to comply with previously described bioassays (Audsley *et al*., 2001). Control slugs were injected with the respective amount of DMSO.

Terrestrial gastropods do not always feed on a regular daily basis. Therefore, the animals used in the bioassay were deprived of food for 96 hours before the bioassay took place, in order to ensure that they were at a similar physiological state. The starved animals were weighed, and then anaesthetised by being placed on ice for 10-15min. Subsequently, slugs were treated by injections into the haemocoel using a Hamilton 10μL syringe. After being injected, the slugs were placed in individual pots containing a glass vial with artificial diet of known wet weight. The individual pots were kept under rearing conditions described above. Slugs were weighed again at the end of the experiment. Daily food consumption was monitored for four consecutive days and was calculated by subtracting dry weight of remaining diet (dried to constant weight at 60^{o}C) from converted dry weight of the diet given to individual slugs. Dry weight of diet given was determined using a conversion factor calculated from the correlation between the wet weight and the dry weight of a range of diet standards.

E. 2. Results and Discussion

GNA was detected in all parts of the digestive tract of the animals, including the faeces (fig. 11), therefore suggesting that it can pass through the digestive tract of slugs, and remain largely intact. These results also indicate the presence of potential binding sites for GNA (e.g. glycoproteins) in the digestive tract of the slugs, but further studies are needed in order to verify this.

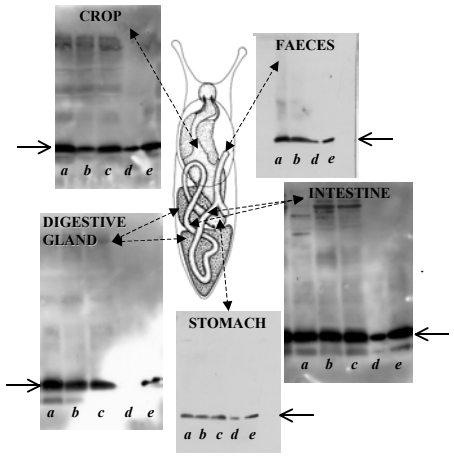

Figure 11. **Presence of GNA in polypeptides (indicated by the arrows) extracted from several parts of the digestive tract and faeces of *Arion subfuscus* after feeding slugs with GNA containing artificial diets. Samples from slug tissues were loaded on lanes a,b,c whereas GNA markers of 10 and 50ng were loaded on lanes d and e respectively. Samples were prepared and analysed by SDS-PAGE (15% acrylamide gels). Gels were blotted onto nitrocellulose and probed with primary anti-GNA antibodies, secondary peroxidase-labelled secondary antibodies, followed by treatment with ECL reagents and exposure to X-ray film. Diagram of the slug digestive system: after G.M. Barker.**

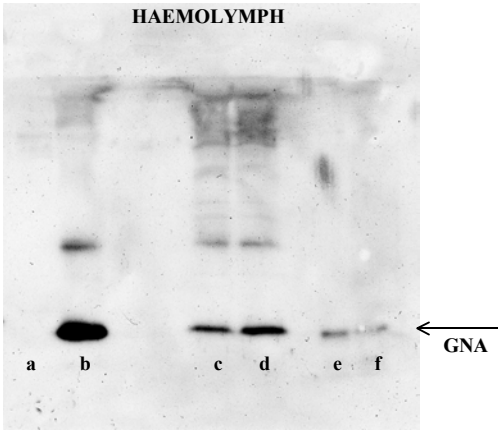

Figure 12. **Presence of GNA in haemolymph of *Arion subfuscus* after feeding slugs with GNA containing artificial diet. Samples from slug tissues were loaded on lanes b, c, d whereas GNA markers of 10ng were loaded on lane and e and f. Peptides from control haemolymph were loaded on lane a.**

In invertebrates, the presence of glycoproteins in the digestive system which bind to lectins, specifically GNA and concanavalin A, *in vitro* has been confirmed in our lab with immunolocalisation studies in the tomato moth (*L. oleracea*) larvae (Fitches *et al*., 2001).

GNA was also detected in the haemolymph of GNA fed slugs (fig. 12), irrespective of the species tested, indicating that GNA is resistant to gut proteolysis, and that it is able to cross the gut epithelial cells and to be transported into the circulatory system of terrestrial gastropods.

In *D. reticulatum*, GNA was detected in the haemolymph of 79% (n=38) of the GNA-fed animals (n=48). In contrast, in *A. subfuscus*, GNA was detected in the haemolymph of only 23% (n=3) of the GNA-fed animals (n=13). The above results support the hypothesis that GNA could indeed be used as a carrier molecule for biologically active peptides in terrestrial pest gastropods. In *A. subfuscus*, GNA was detected in the haemolymph of fewer animals as compared with *D. reticulatum*. This result might be due to the substantially larger size of *A. subfuscus*, compared to *D. reticulatum*; this in its turn, could affect the scale of dilution of GNA in the haemolymph of the slugs.

Injection of 5μg Manse-AS per 100mg body weight into the haemocoel of slugs had no effect on the food consumption when compared with control slugs (Table I). Similarly, the weight increase of the slugs injected with Manse-AS and that of the control slugs was similar (23.48 ± 7.37 mg and 18.85 ± 7.28 mg, respectively). These results indicate that Manse-AS has no biological action as a feeding inhibitor in terrestrial slugs, and therefore this particular fusion protein, GNA/Manse-AS, may not be sufficiently effective to be used as a terrestrial slug control agent.

Table I. Cumulative food consumption over 96 hours (mean ± standard error) in slugs injected with synthetic Manse-AS (5μg/100mg wet weight). Control slugs were injected with the respective amount of DMSO.

	Cumulative food consumption (mg)			
	24 hours	48 hours	72 hours	96 hours
Injection of 5μg/100mg Manse-AS (n=45)	14.70±2.27	22.8 0±2.87	35.00±3.64	47.80±4.19
Injection of 1μl/100mg DMSO (n=45)	11.26±1.11	18.03±1.50	30.42±2.11	42.22±2.80

F. Investigation of the egg-laying behavioural pattern of the grey field slug, *D. reticulatum*, under laboratory conditions with the aim to develop a reliable bioassay(s) to test potential egg laying inhibitors

The grey field slug, *D. reticulatum*, presents two major breeding seasons in the field each year with the juvenile slugs to emerge in late spring and in late autumn, although *D. reticulatum* eggs may be found in the field throughout the whole year (South, 1992). However, under constant favourable environmental conditions, i.e. laboratory conditions, this pest slug is able to reproduce and egg lay continuously (South, 1992).

In our laboratory, we decided to develope a specific bioassay in order to be able to assess reliably molecules that might have a potential as egg laying inhibitors for the grey field slug *D. reticulatum*.

F. 1. Experimental protocol

Under the constant laboratory conditions in our breeding unit, individual *D. reticulatum* slugs exhibit a considerable variability in their rate of growth and reproductive maturity. In particular we observed that slugs may start to be reproductively active as early as 6 weeks after hatching, provided that they are kept in groups (personal observations). Therefore, in order to acquire virgin slugs for our experiments, we separated the slugs individually when they reached the 6th week of their development (under laboratory conditions). The separated individual slugs were grouped once more (in sets of 10 individuals) at different times after hatching (i.e. 8, 9, 10, 11, 12, and 13 weeks of age) for either one or two weeks (namely the "mating period"). Thereafter, the slugs were separated individually again, and their egg-laying activity was monitored. Our results showed that 11 weeks of age is the earliest age at which virgin slugs start exhibiting mating behaviour in the presence of other slugs. Additionally, only about 50% of the slugs that were treated as indicated above would finally attain complete female reproductive activity and produce viable eggs.

F. 2. Results and Discussion

Individual fecundity, under the experimental conditions described above, reached a maximum of 109 eggs per individual slug.

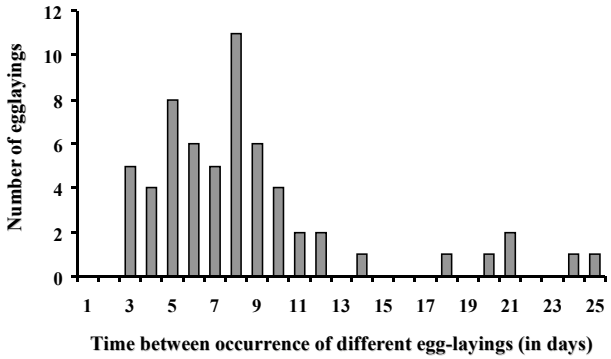

Figure 13: Temporal representation of the total egg-layings produced by individual slugs D. reticulatum (n=38) under laboratory conditions and under the specific experimental protocol described above (for more information see notes in the section F. 1. 1.)

This value appears to be lower than those reported previously for this slug species by Hunter (1978; n=200) and by Port and Port (1986; n=260 to 500). However, under our experimental conditions the slugs were allowed to mate only for a limited time (either one or two weeks) before their egg laying behaviour was recorded. Thus, it is possible that our experimental protocol minimised the total number of possible matings between individual slugs, therefore affecting the total number of eggs produced by each slug.

The mean number of eggs per egg-laying was 45.5 ± 5.7; each individual slug may egg-lay several times (ranging from 1 to 11) with the majority of the slugs producing more than one batch of eggs. The egg laying activity of the animals may last for several weeks after the first egg-laying, and the hatches of eggs produced by each individual slug may be laid at variable intervals ranging from 3 to 21 days. However, the majority of the subsequent egg hatches produced occurred at intervals ranging from 5 to 9 days (fig. 13).

We analysed further the egg-laying behavioural pattern in individual slugs by taking into account the relationship between their reproductive index [(RI=number of egg-layings / total number of days exhibiting egg-laying behaviour)*100] and the time interval between the occurrence of the earliest and the latest egg-laying of each slug (fig. 13).

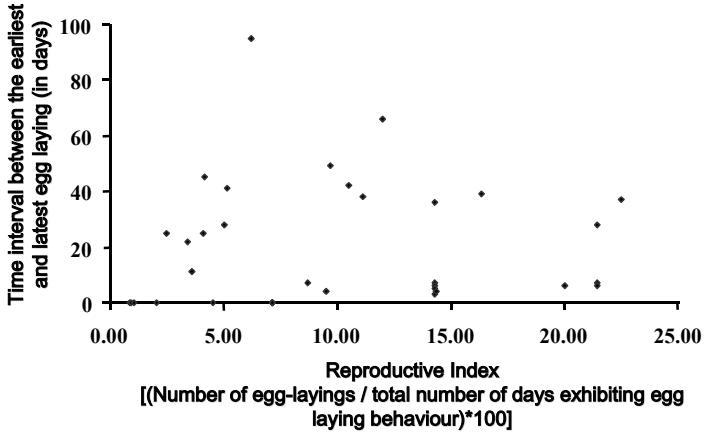

Figure 14: **Scattergram of the different reproductive indexes of individual slugs Deroceras reticulatum under laboratory conditions and under the specific experimental protocol described above (for more information see notes in the section F. 1. 1.)**

The analysis showed that the egg laying behaviour of the grey field slug, *D. reticulatum*, is complex and highly variable since there was no particular pattern of either the number and/or the timing of the egg-layings produced by each individual slug (fig. 14). It appears, therefore that the grey field slug employs a rather opportunistic egg-laying behaviour.

The experimental trials performed provided us with essential information on the egg-laying behavioural patterns of *D. reticulatum*, and they gave a good basis upon which we designed a specific experimental protocol for assessing different molecules for potential egg-laying inhibitory activity (see below).

G. The potential of the *Lymnaea* neuropeptide Y as an egg-laying inhibitor in the terrestrial slug *D. reticulatum*

Neuropeptide Y (NPY) is known to participate in many aspects of regulation of growth and reproduction in vertebrates. Hoewever, NPY is also present in invertebrates (Leung *et al*., 1992; de Jong-

Brink *et al*., 2001), and recently, it has been shown that it may play an important role as a reproduction and growth regulator in invertebrates including some freshwater snails (*Lymnaea stagnalis*: de Jong-Brink *et al*., 1999; de Jong-Brink *et al*., 2001). De Jong-Brink et al. (1999) showed that by increasing the *Lymnaea* Neuropeptide Y (LyNPY) titre in the blood of the snails (either by injection or by slow release pellets) the egg laying activity of the animals was prevented (by 99%). This specific effect of LyNPY was dependent on the exact reproductive status of the snails: when delivered to animals that have recently laid eggs it suppressed their egg-laying activity more, compared with animals that have for longer periods without laying any eggs (de Jong-Brink *et al*., 1999). De Jong-Brink *et al*. (2001) compared the molecular structure of NPYs in invertebrates (including the terrestrial snail *H. aspersa*), and they showed that the molluscan and the arthropod NPYs appeared to be synthesised from a pro-hormone that was similar to that of vertebrate NPY, and they suggested that they should be considered as real invertebrate homologs of NPY.

The presence of NPY-like molecules in terrestrial snails (Leung *et al*., 1992; de Jong-Brink *et al*., 2001) indicated that this peptide might also have potential as a reproductive regulator in terrestrial pulmonates. In our laboratory we had developed a reliable bioassay in order to assess the egg-laying inhibitory activity in terrestrial slugs, and we decided to synthesise LyNPY (in collaboration with the biotechnology company Pepceuticals Ltd.), and subsequently test its biological activity, if any, on the egg-laying activity of *D. reticulatum*.

G. 1. Methods and Materials

G. 1. a. Biological material

All *D. reticulatum* slugs used in the experiments were reared in our laboratory under controlled environmental conditions (20^{o}C, L:D 14:10, R.H. 95%), and they were of known age and physiological status.

G. 1. b. Synthesis of *Lymnaea* neuropeptide Y (LyNPY)

Lymnaea neuropeptide Y was custom synthetised using solid phase methodology (Fmoc procedure) by the Pepceuticals Ltd., Beaumont house, 72 Boston rd, Leicester, UK. The purity (>95%) of the synthetic peptide was confirmed by combined HPLC and MALDI/TOF Mass Spectrometry analyses.

G. 1. c. Experimental procedure for injecting synthetic LyNPY

Synthetic LyNPY was dissolved in physiological saline and injected into the haemocoel of the animals at a ratio of 1μM per individual slug in order to comply with previously described bioassays (de Jong-Brink *et al*., 1999). Control slugs were injected with the respective amount of physiological saline. The slugs were injected daily in order to mimic the effects of a slow release pellet since it has been known that the titre of LyNPY needs to remain at a certain level in the blood of the animals in order to exert its effects (de Jong-Brink *et al*., 1999).

The animals used in the bioassay remained grouped in growth boxes for 6 weeks after hatching, thereafter being separated for a further 5 weeks (fig. 15). At the end of their 11th week of age they were grouped into sets of 10, and were kept in reproductive boxes for a further two weeks in order to be allowed to

mate (fig. 15). The slugs were separated again at the end of this two-week "mating period", and they were placed into individual pots. The pots were checked twice daily for presence of any eggs, and only individual slugs that exhibited egg-laying activity were used for injections with either synthetic LyNPY or physiological saline.

G. 2. Results and Discussion

The results obtained, shown in figure 15, indicate that by injecting a relatively small quantity of the synthetic LyNPY (i.e. 1μM) the egg-laying activity of *D. reticulatum* may be inhibited by 40%.

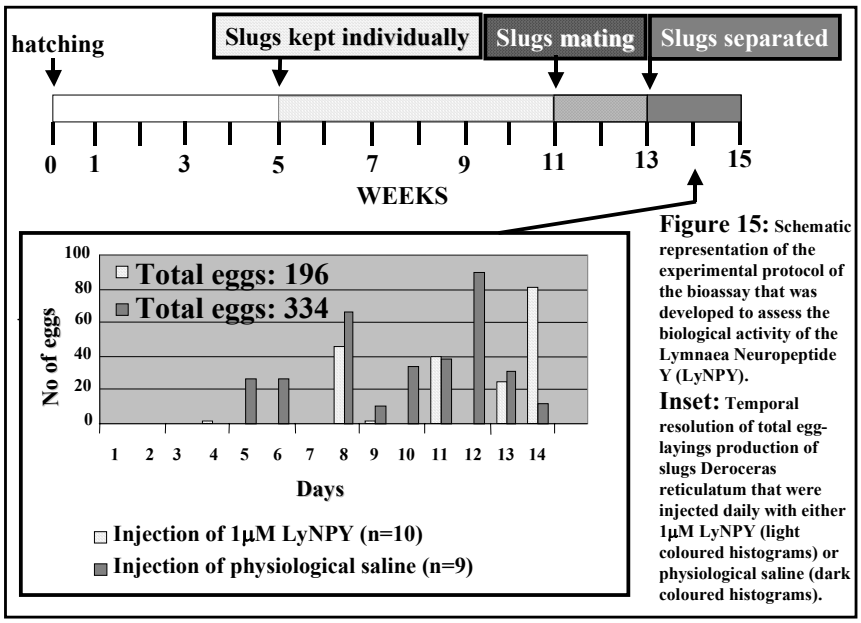

Figure 15: Schematic representation of the experimental protocol of the bioassay that was developed to assess the biological activity of the Lymnaea Neuropeptide Y (LyNPY). **Inset:** Temporal resolution of total egg-layings production of slugs Deroceras reticulatum that were injected daily with either 1μM LyNPY (light coloured histograms) or physiological saline (dark coloured histograms).

This inhibitory effect of LyNPY of egg-laying in terrestrial slugs is in accordance with the role of the peptide as a reproductive and growth regulator reported previously for pulmonate molluscs, albeit the freshwater representatives (de Jong-Brink *et al*., 1999). However, the degree of this inhibitory effect in terrestrial pulmonates (40%) appears to be significantly lower than that reported for the freshwater snails (>99%); this might be due to the fact that, although NPY molecules derived from terrestrial and freshwater representatives display a significant homology with the vertebrate NPY, the overall homology between the individual invertebrate sequences is not great (de Jong-Brink *et al*., 2001).

In vertebrates, the inhibition of reproduction by NPY is due to its inhibiting effect on release of reproductive hormones from the hypothalamus and the pituitary (Kalra and Kalra, 1996 cited in de Jong-Brink *et al*., 1999). As regards its action mode in invertebrates, de Jong-Brink *et al*. (1999) presented evidence that the physiological changes that accompany the delivery of LyNPY result from an effect of the peptide on central neuroendocrine cells that are involved in the control of reproduction of growth in these animals. Further investigations of the anatomical presence of Neuropeptide Y in terrestrial pulmonate

molluscs, as well as its exact physiological role in the growth and reproduction of these animals, is needed in order to evaluate fully its potential as a reproductive and growth inhibitor for terrestrial pest slugs and snails.

H. The potential of the FMRFamide peptide as an inhibitor of the reproductive maturation of the grey field slug *D. reticulatum*

To our knowledge, no research has yet been published in which evidence is presented for the existence of (neuro)regulators that stimulate the synthetic activity of the Dorsal Bodies Cells (DBCs). However, there has been some advance in relation to the discovery of neuropeptides (particularly FMRFamide-like peptides), which may be involved in the inhibitory control of the DBCs. In particular, FMRFamide is a peptide that has been implicated in the inhibitory regulation of protein synthesis by DBCs maintained *in vitro* (Mounzih and Griffond, 1988; Griffond and Mounzih, 1989). This peptide is localised only in certain groups of cerebral green cells (Ce-GCs), particularly those of medium size (40-50μm) (Marchand *et al*., 1991), and is also present in type II axons that run from Ce-GCs and form synapse like structures with DBCs (Griffond and Mounzih, 1990; Marchand *et al*., 1991). Furthermore, the presence of FMRFamide and of 2 FMRFamide-like substances in the connective tissues containing the DBCs was confirmed using combined HPLC and RIA methodology (Mounzih and Griffond, 1992).

Moreover, there is also evidence that supports the involvement of FMRFamide peptides in the control of the male reproductive behaviour. Male copulatory behaviour has been studied extensively in the freshwater snail *L. stagnalis*, and it has been shown that it is controlled by a neuronal network consisting of five clusters of central neurons located in the anterior and ventral lobes of the right cerebral ganglion. These neuronal clusters are interconnected, and send an axon into the penis nerve (Smit *et al*., 1992 cited in van Kesteren *et al*., 1995). The penis nerve is the sole nerve that innervates the penis complex, its associated muscles, the vas deferens and the prostate gland. Most of the above cells are peptidergic. Application of direct Matrix-Assisted Laser Desorption Ionization Mass spectrometry (MALDI-MS) to neurons and nerves has led to the identification of some of the numerous peptides, including FMRFamide, that are produced in the neuronal network controlling male copulatory behaviour in freshwater snails (e.g. APGWamide, conopressin, neuropeptide tyrosine, FMRFamide and related peptides, various myomodulins, *Lymnaea* inhibitory peptide and pedal peptide, Small Cardioactive Peptides: SPC A and B) (van Kesteren *et al*., 1995; van Golen *et al*., 1995; Dreisewerd *et al*., 1997).

The information cited above indicated that FMRFamide might play an important regulatory role in the process of the reproductive maturation of terrestrial pulmonates. Moreover, the effects of FMRFamide *in vivo* have not been tested so far in any terrestrial pulmonate gastropod. Therefore, it was decided that the effects of FMRFamide on the reproductive maturity of the grey field slug *D. reticulatum* should be investigated in order to evaluate the potential of this particular peptide as an inhibitor of reproduction of this pest slug.

H. 1. Methods and Materials

H. 1. a. Biological material

All *D. reticulatum* slugs used in the experiments were reared in our laboratory under controlled environmental conditions (20^{o}C, L:D 14:10, R.H. 95%). After hatching, the slugs used in the bioassay remained grouped in growth boxes until they reached their 6th week of age, thereafter being separated in individual pots.

H. 1. b. Experimental procedure for injecting synthetic FMRFamide and evaluating its effects on the reproductive maturity of the slugs

Synthetic FMRFamide was obtained from Sigma UK and was dissolved in physiological saline. FMRFamide was injected into the haemocoel of the animals at a ratio of 0.1mM per individual slug once a week (FMRFamide slugs; n=12). As a control, a second group of slugs were injected with the respective volume of physiological saline (=21μl) (PS slugs; n=12). In order to determine whether the reproductive maturity of slugs might be affected by the stimuli of the weekly injection we introduced a third group of slugs which were not subjected to any injections (Control slugs; n=23).

At the end of their 16th week of age the animals were weighed, and then anaesthetised by being placed on ice for 10-15min. Subsequently, the several parts of their reproductive system (i.e. female and male reproductive tracts, penis, albumen gland, hermaphroditic gland) were dissected on ice under a stereomicroscope, and the respective reproductive indexes were calculated [($W_{\text{part of reproductive system concerned}}$/Wet weight)*100].

H. 2. Results and Discussion

Results obtained from the control group indicate that, in the grey field slug *D. reticulatum*, the hermaphroditic gland (i.e. gonad) is one of the first organs of the reproductive system to develop, a result that is in accordance with the work of Runham and Laryea (1965). In particular as regards the interrelationships between the growth rates of the different parts of the slug reproductive system, it appears that the growth of the hermaphroditic gland is negatively correlated with the one of the reproductive tract, including this of the albumen gland (figs. 16, 18). Therefore, the more the slugs progress into the maturation of their female reproductive system, the higher the reproductive indexes of the albumen gland and the female reproductive tract are; in contrast, the reproductive index of the gonad is becoming lower (figs. 16, 18). The albumen gland and the female reproductive tract appear to mature in parallel, thus their correlation is positive (fig. 19).

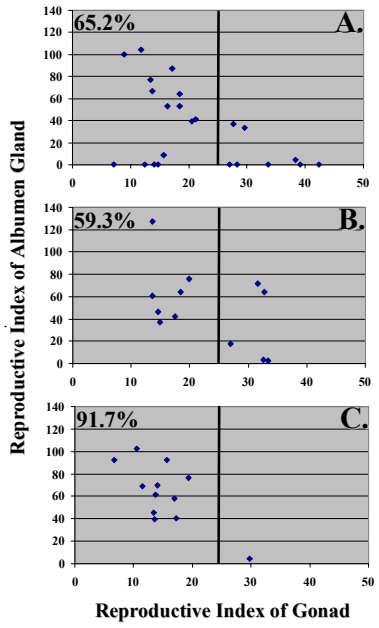

Figure 16: **Graphical representation of the relationship between the Reproductive Indexes of the Gonad and of the Albumen Gland in the i) control slugs (A), ii) slugs injected with 0.1mM FMRFamide weekly and iii) slugs injected with physiological saline weekly (C).**

Initially, we compared the mean of the different reproductive indexes between the physiological saline injected and the FMRFamide injected slugs. The comparisons revealed that the FMRFamide injected slugs had significantly higher RI_{GONAD} (fig. 17), indicating that this particular group of slugs had a slower rate of reproductive maturity.

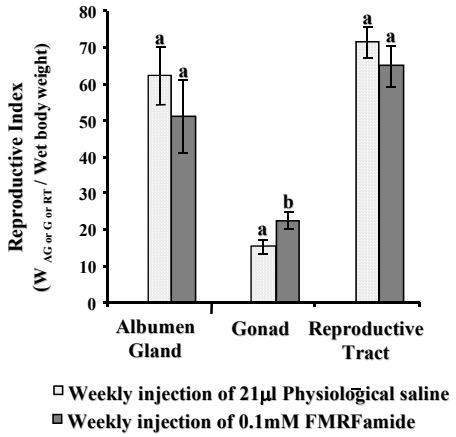

Figure 17: **Mean ± Standard Error of the reproductive indexes of the different parts of the reproductive system in slugs *Deroceras reticulatum* injected weekly with 0.1mM FMRFamide (dark coloured histograms) or physiological saline (light coloured histograms). Histograms sharing a common letter do not differ significantly but do differ from histograms with a different letter (P<0.05).**

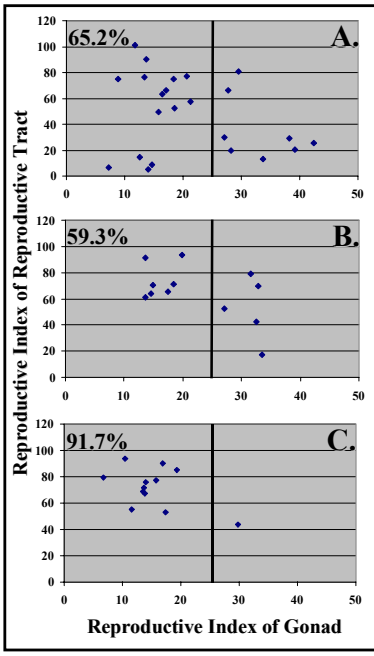

Figure 18: **Graphical representation of the relationship between the Reproductive Indexes of the Gonad and of the Reproductive Tract in the i) control slugs (A), ii) slugs injected with 0.1mM FMRFamide weekly and iii) slugs injected with physiological saline weekly (C).**

When we analysed the interrelationships between the different reproductive indexes in individual slugs it was revealed that the animals that were injected with physiological saline attained their reproductive maturity more uniformly and more rapidly than the slugs in the control group (figs. 16, 18; 91.7% of the animals had a RI_{GONAD}<25 compared with only 65.2% from the control group), indicating that the act of injecting might have invoked a tactile stimulatory effect on the slugs.

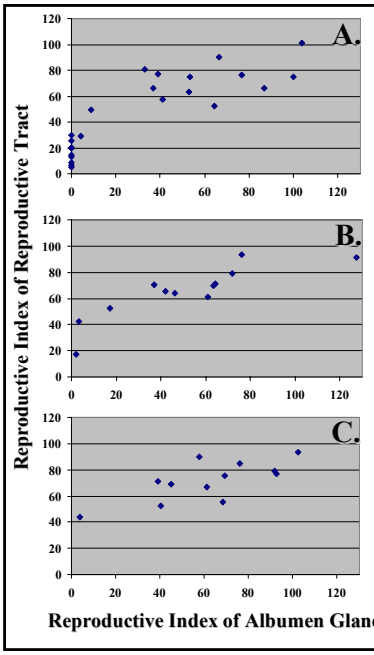

Figure 19: **Graphical representation of the relationship between the Reproductive Indexes of the Reproductive Tract and the Albumen Gland in the i) control slugs (A), ii) slugs injected with 0.1mM FMRFamide weekly and iii) slugs injected with physiological saline weekly (C).**

In contrast, the rate of reproductive maturity of slugs that were injected with FMRFamide weekly was less uniform and slower than the animals that were injected with physiological saline, and therefore it was

closer to that of the control slugs (figs. 16-18). This slower rate of reproductive maturity of the slugs that were injected with FMRFamide could be due to an inhibitory effect of the peptide on the function of the main female reproductive hormone, the Dorsal Body Hormone. Such action is in accordance with previously reported *in vitro* results that FMRFamide might be involved in the nervous inhibitory control of Dorsal Bodies Cells (Mounzih and Griffond, 1988; Griffond and Mounzih, 1989). These were analysed in more detail in a previous section (section C.3). It appears therefore that FMRFamide might hold a potential as a reproductive regulator for the grey field slug *D. reticulatum*; nonetheless, futher research is needed in order to quantify the possible relationship between this peptide and the main female gonadotropic hormone in terrestrial slugs.

I. The potential of a conotoxin as a feeding inhibitor and as a molluscicide of the grey field slug *D. reticulatum*

It has been long recognised that naturally occurring toxins (either animal or plant derived) are good candidates for the development of novel and highly efficient pesticides, mainly because:

i) they are highly specific, and

ii) they usually target crucial parts of the underlying biochemical pathways and mechanisms at the cell level.

The cone snails (i.e. *Conus* spp.) are considered to be the most venomous of all molluscs (Olivera *et al*., 1991). All *Conus* species are believed to be predatory. Each *Conus* species has a venom with a distinct pharmacological profile and it usually targets a particular type of prey, that could be either a fish, a marine worm or a marine mollusc (Cruz *et al*., 1985; Olivera *et al*., 1991). These venoms are highly complex and they contain a large number of biologically active peptides, namely conotoxins or conopeptides. It is estimated that the venom of each *Conus* species includes between 50 to 200 conotoxins (McIntosh *et al*., 1999). These (neuro)toxic conotoxins are cysteine-rich proteins, usually small in size, and typically highly selective to either vertebrate or invertebrate tissue depending on the particular type of the prey that each *Conus* species attacks. The biochemical uniqueness of these conotoxins arises from their small size and their highly rigid tertiary structure (which is due to the disulfide bridges that form between the cysteine groups in the molecule). These characteristic structural features (i.e. number of the cysteines and the particular type of the scaffolding among them) are used to classify the conotoxins in distinct classes; each one of these classes has been shown to follow a particular mode of action within the cell (McIntosh *et al*., 1999).

We concentrated our literature search on the mollusc-paralysing conotoxins, and specifically on those that were known not to exhibit any paralytic effects in either other invertebrates (i.e. insects) or vertebrates (Fainzilber *et al*., 1991; Spira *et al*., 1993). These conotoxins are of particular interest for us as they might hold a potential for the development of highly selective control agents of terrestrial gastropod pests. A particular conotoxin (namely TxIA) was chosen as a candidate for further investigation in relation to any

biological activity it might have had either as a feeding inhibitor and/or as a molluscicide of the grey field slug *D. reticulatum*.

I. 1. Methods and Materials

I. 1. a. Biological material

All *D. reticulatum* slugs used in the experiments were reared in our laboratory under controlled environmental conditions (20^{o}C, L:D 14:10, R.H. 95%), and they were of known physiological state and age.

I. 1. b. Synthesis of the TxIA conotoxin

The TxIA conotoxin was custom synthetised using solid phase methodology (Fmoc procedure) by Pepceuticals Ltd., Beaumont house, 72 Boston rd, Leicester, UK. The purity (>95%) of the linear peptide was confirmed by combined HPLC and MALDI/TOF Mass Spectrometry analyses. Thereafter, the peptide was cyclised, in order for the disulfide bridges to be formed, under air oxidative conditions. Consequently, the end product comprised a mixture of peptides, all analogues of the same peptidic sequence. It was expected that the most energetically favourable confirmation of the cyclised peptide was the same as the one found in the naturally occurring peptide.

I. 1. c. Experimental procedure for injecting synthetic TxIA conotoxin

Synthetic TxIA was dissolved in dimethyl sulfoxide (DMSO), due to the hight hydrophobicity of this molecule, and was injected into the haemocoel of the animals at a concentration of 20mM per 100mg wet weight (Conotoxin injected slugs; n=90). As a control, a second group of slugs were injected with the respective volume of DMSO (1μl per 100mg of wet weight) (DMSO injected slugs; n=90). In order to assess whether injection in itself provoked any stimulatory or inhibitory effect on the animals a third group of slugs were subjected to sham injections (Control slugs; n=90).

I. 1. d. Experimental procedure for determining feeding activity

The slugs used in this bioassay were deprived from food for 72 hours, before the bioassay took place, in order to ensure that they all were at a similar physiological state. The starved animals were weighed, and then anaesthetised by being placed on ice for 10-15min. Subsequently, slugs were treated by injections into the haemocoel using a Hamilton 10μL syringe. After being injected, the slugs were placed in individual pots containing a glass vial with artificial diet of known wet weight. The individual pots were kept under the rearing conditions described above. Slugs were weighed again each time they were provided with fresh food, namely every 3-4 days, and at the end of the experiment. The food consumption was monitored every 3-4 days. Food consumption was calculated by subtracting dry weight of remaining diet (dried to constant weight at 60^{o}C) from converted dry weight of the diet given to individual slugs. Dry weight of diet given was determined using a conversion factor calculated from the correlation between the wet weight and the dry weight of a range of diet standards. Data were statistically analysed with ANOVA, and pair comparisons between the different experimental groups were performed by Fisher's test.

I. 2. Results and Discussion

Only juvenile slugs were used in the conotoxin experimental trials as we had restrictions in terms of the amount of the synthetic peptide available, as well as the volume of the DMSO that could be injected into the animals. The mean initial weight of the control, the conotoxin injected and the DMSO injected slugs was 94.6±6.51, 98.6±6.12 and 94.15±5.91 mg, respectively.

The results obtained show that the injection of 20mM of TxIA conotoxin into the haemocoel of juvenile *D. reticulatum* had adverse effects on the food consumption and consequently the weight, as well as the survival of the animals (figs. 20-22).

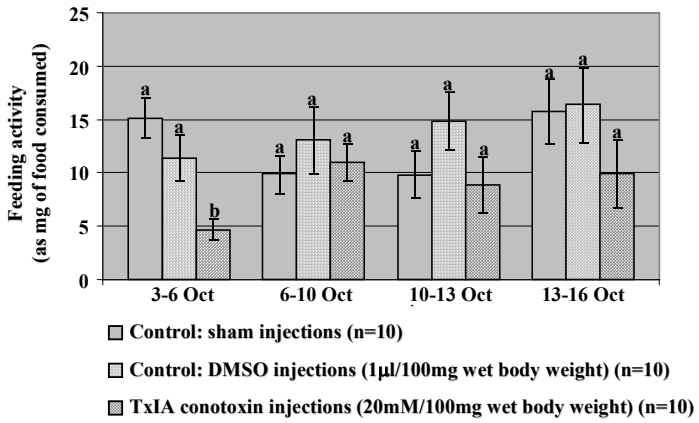

Figure 20: **Food consumption (expressed as mg of artificial diet consumed) of juvenile slugs *Deroceras reticulatum* during a 15 days time period following either sham injections ( ), injection with DMSO( ) or injections with TxIA conotoxin ( ).**
Histograms sharing a common letter do not differ significantly but do differ from histograms with a different letter (P<0.05).

Nevertheless, these effects were more obvious during the first three to four days after the injections took place (figs. 20, 22). In particular, with food consumption, it appears that following the initial significant decrease that took place post-injection, the conotoxin injected slugs resumed their feeding activity at levels comparable to these of the control groups (fig. 20). Similarly, after the initial three to four days post-injection, the weight of the conotoxin injected slugs was significantly reduced (96.67±8.03 mg; mean weight reduction of –6.1±4 mg) compared with the sham injected animals (107.7±6.18 mg; mean weight gain of 13.1±2.71 mg) (fig. 21).

As expected, the final weight of the animals that were injected with DMSO was significantly affected (98.6±5.76 mg; mean weight gain of 2.07±3.6 mg) in comparison with the control slugs, therefore indicating that DMSO may account, albeit partly, for the total weight reduction that the slugs injected with the TxIA conotoxin suffered (fig. 21). After the 5th day post-injection, however, the weight changes of the different experimental groups were less dramatic (data not shown).

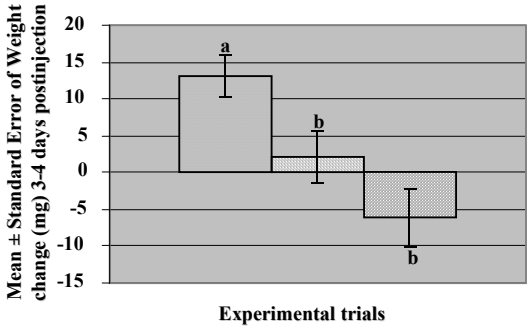

Figure 21: Mean ± Standard Error of the weight change of juvenile slugs *Deroceras reticulatum* during the initial 3-4 days following either control sham injections (□), injection with DMSO(□) or injections with TxIA conotoxin (▨). Histograms sharing a common letter do not differ significantly but do differ from histograms with a different letter (P<0.05).

□ Control: sham injections (n=30)

□ Control: DMSO injections (1μl/100mg wet body weight) (n=30)

▨ TxIA conotoxin injections (20mM/100mg wet body weight) (n=30)

The adverse effects of TxIA conotoxin on juvenile slugs *D. reticulatum* were also evident in the survival data of the experimental trials (fig. 22). It appears that, this particular conotoxin has an acute lethal effect, albeit mild, as 20% of the slugs injected with TxIA died either the same day or the day following the administration of the toxin. Thereafter, the mortality rate of the injected slugs was less sharp; nevertheless, 9 days after the injections took place 47% of the slugs injected with TxIA conotoxin were dead compared with only 27% and 17% from the DMSO injected slugs and from the control group, respectively (fig. 22).

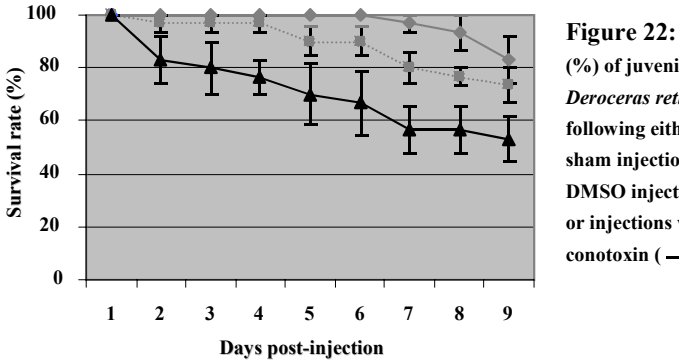

Figure 22: Survival rate (%) of juvenile slugs *Deroceras reticulatum* following either control sham injections (—◆—), DMSO injections (·····■·····), or injections with TxIA conotoxin (—▲—).

—◆— Control: sham injections (n=30)

·····■····· Control: DMSO injections (1μl/100mg wet body weight) (n=30)

—▲— TxIA conotoxin injections (20mM/100mg wet body weight) (n=30)

The above results indicate that this particular conotoxin (TxIA) has a biological action against terrestrial slugs, although the exact physiological mechanisms underlying this are not as clear. Hasson *et al*. (1993) investigated the mechanism of the action of TxIA and TxIB (which is similar to TxIA) in *in vitro* cultures of neurons isolated from the marine slug *Aplysia*, and they found that the conotoxins cause action potential broadening by prolonging the voltage-gated sodium currents, either by slowing the rate of sodium current inactivation or by activating silent sodium channels. Hasson *et al*. (1993) used naturally occurring conotoxins, and as a result the concentrations at which the conotoxins exerted their biological activity were very low (0.25-0.5μM). However, in our experiments, we performed our experimental trials in the presence of a very high concentration (20mM) since it was known that chemically synthesised conotoxins might exhibit reduced biological activity compared with the natural molecules (Bondebjerg *et al*., 2003).

It should be mentioned however, that these adverse effects of TxIA on *D. reticulatum*, followed a single administration of the conotoxin, and it may be possible that the TxIA effects might have been dramatically more potent if the conotoxin was delivered to the slugs more systemically, for example with a slow release pellet technology.

It is also important to emphasize that evidence reported previously indicates that the TxIA conotoxin exhibits strong paralytic activity in molluscs, with no paralytic effects on arthopods and vertebrates (Hasson *et al*., 1993). In view of this high specificity of the TxIA conotoxin its potential as a highly specific terrestrial slug control agent is enhanced.

ACKNOWLEDGEMENTS

We would like to thank Mr Phil Mason and Miss Alison Pennington for their significant contribution in the laborious maintenance of the slug breeding unit. Also, we would like to thank Mr Mark Sykes for his valuable help in the MALDI/TOF-MS experimental work and the synthesis of the *Lymnaea* Neuropeptide Y and the TxIA conotoxin. The contribution of Drs Rob Weaver, Neil Audsley and Elaine Fitches in the RP HPLC and RIA for immunoreactive ecdysteroids, Manse-AS ELISA and Western blotting for GNA, respectively is also acknowledged. Finally we wish to thank HGCA and DEFRA for supporting this important strategic research.

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