# THE RELATIONSHIP BETWEEN MESOCEREBRAL ACTIVITY AND SEXUAL AROUSAL IN THE SNAIL, *CORNU ASPERSUM*

Maral Tajerian

Department of Biology
McGill University
Montreal

August 2007

A thesis submitted to McGill University in partial fulfillment of the degree of
Master of Science

# TABLE OF CONTENTS

# ABSTRACT

The courtship behavior of the pulmonate snail *Cornu aspersum* comprises an intricate sequence of acts followed by dart shooting. This study aims at comparing mesocerebral electrical activity in ganglia removed from snails belonging to three stages of sexual arousal: Non-excited snails, pre dart shooters, and post dart shooters. The groups were compared using measurements of spontaneous mesocerebral activity and responses to simultaneous stimulation of six nerves. Dart shooters showed the highest spontaneous activity, followed by the pre dart shooters and finally, non-excited snails. Nerve stimulation increased activity only in non-excited snails. Cluster analysis failed to show a significant change in the number of active units before and after nerve stimulation. These results show that mesocerebral activity is correlated with the behavioral stages of courtship, and is influenced by input from the stimulated nerves, thus linking the ethology and physiology of mating in this species.

# RESUMÉ

Le comportement d'accouplement de l'escargot pulmonate, soit la *Cornu aspersum,* est composé d'un rituel établit suivi du lancement du dard. Cet étude vise à faire une étude comparative entre les différent niveaux d'activité électrique mésocérébrale perçus dans les ganglions extrait d'escargots ayant atteint l'un des trois niveaux d'excitation sexuelle, soit les escargots: non-excités, pré-lanceurs de dards et post-lanceur de dards. De ces échantillons, le niveau spontané d'activité mésocérébrale et l'effet de la stimulation simultanées de six nerfs ont été comparés. L'échantillon composé des post-lanceurs de dards ont démontré des niveaux spontané d'activité mésocérébrale les plus élevés parmi les trois; les échantillons composé de pré-lanceurs de dards et d'escargots non-excités ont démontrés des niveaux respectivement inférieurs à celle-ci. Alternativement, les stimulant nerveux augmentent uniquement l'activité mésocérébrale des escargots non-excités. Malgré ces indicatifs statistiquement significatif, l'analyse de groupement a échouer à la tâche d'identification d'un changement significatif d'unités actives avant et après la stimulation nerveuse. Ces résultats démontrent qu'il y a effectivement un corrélation entre l'activité mésocérébrale et les différents comportements d'accouplement associé a chacun des niveau d'excitation; en plus d'être affecté par l'influx sensoriel provenant des nerfs stimulés, ainsi liant l'ethologie et la physiologie au processus d'accouplement de l'espèce.

# LIST OF FIGURES AND TABLES

# LIST OF ABBREVIATIONS AND SYMBOLS

ALN: Anterior lip nerve

APGWamide: Ala-Pro-Gly-Trp-NH2

CPG: Central pattern generator

FMRFamide: Phe-Met-Arg-Phe-$NH_2$

LTN: Left tentacle nerve

MLN: Medial lip nerve

PCA: Principal component analysis

PLN: Posterior lip nerve

PN: Penis nerve

RCPN1: Right cutaneus pedal nerve number 1

RCPN2: Right cutaneus pedal nerve number 2

RCPN3: Right cutaneus pedal nerve number 3

RTN: Right tentacle nerve

# ACKNOWLEDGEMENTS

This thesis was the result of two years of work at the excellent department of Biology at McGill University, which provided me with a stimulating research and learning environment. I do not think it is possible to overstate my gratitude to my thesis supervisor, Professor Ronald Chase, for his support and guidance throughout my studies. His enthusiasm and scientific curiosity were contagious, and I owe most of what I learned here to him.

I would also like to thank my supervisory committee members, Professor Rudiger Krahe and Professor Gerald Pollack, who were always ready to help with their invaluable advice and constructive criticism.

Emily Darbyson and Benoit Valin made my work at the laboratory a much more pleasant experience. Apart from their friendship, they were always ready to help with the editing and the translation of the abstract into French. I am also grateful to all my lab mates throughout these two years. Their support and friendship were invaluable.

Finally, I would like to thank my parents, who always believed that I could do anything. None of this would have been possible without their unconditional love and support.

# Chapter 1

# INTRODUCTION

## 1.1 Statement of the research problem

One of the things that the history of science has revealed is that some of the most peculiar behaviors are often the ones that are best understood. The courtship behavior in a number of pulmonate snails is a good example. The common garden snail, *Cornu aspersum* Müller (1774) (previously known as *Cantareus aspersus* and *Helix aspersa*) displays the bizarre ritual of pushing a calcareous dart into its partner's body wall prior to copulation. Consequently, scientists have been studying the courtship behavior, the function of the dart, and the neural control of the different courtship acts in these snails. Following many decades of research, courtship behavior has been accurately documented, the function of the dart is now better understood, and the neural control of courtship and reproduction has been studied. The role of the right mesocerebrum as an integrator of mating behavior has been established based on a body of evidence using various techniques. Nonetheless, a thorough understanding of mesocerebral cell activity during different stages of sexual arousal is still lacking.

The research carried out for this thesis aims at bridging the gap between two different fields: Ethology and neurophysiology. It aims at investigating the differences in mesocerebral cells in snails interrupted at three different stages of sexual arousal: Non-excited snails, pre dart shooters, and post dart shooters. The following criteria are used to compare among the three groups: spontaneous mesocerebral activity and response to various simultaneous nerve stimulations.

The method of choice for recording mesocerebral activity is *in vitro* recording owing to its ease of replication.

## 1.2 Courtship and mating in *Cornu aspersum*

Courtship behavior has been studied in a number of species of land snails (for review, see Chase, 2002). The common garden snail, *Cornu aspersum* (Gastropoda: Pulmonata: Stylommatophora: Helicidae) mates as a simultaneous reciprocal hermaphrodite, both receiving and donating sperm during each mating trial (Tompa, 1984). Its courtship behavior has fascinated scientists for decades because of the bizarre act of dart shooting, when sexually aroused animals push calcareous darts into the body wall of their partners (Hunt, 1979). There have been different hypotheses concerning the function of the dart: Sexual stimulation (Goddard, 1962), a means of species recognition (Webb, 1952), and a gift of calcium (Charnov, 1979; Leonard, 1992). Recently, however, it has been shown that the function of the so called "love dart" is that it provides a large surface area for carrying mucus which increases paternity (Chase and Blanchard, 2006) by triggering a reconfiguration of the copulatory canal, thus increasing the chance of the sperm to be stored instead of digested (Koene and Chase, 1998).

Courtship is initiated after two snails encounter each other and start tentacular, lip, and genital touching, prior to dart shooting; and is finally concluded by copulation. The most detailed account of courtship behavior in

*Cornu aspersum* is that of Adamo and Chase (1988), who categorized the different courtship acts into six stages.

Stage 0: No eversion, the animal has a barely visible genital pore.

Stage 1: The genital atrium starts to protrude.

Stage 2: The penial lobe is moderately everted.

Stage 3: The penial lobe is fully everted, and the penial opening is visible.

Stage 4: Both penial and vaginal lobes are clearly visible, animal exhibits full genital eversion.

Stage 5: There is a distinct bulging of the vaginal lobe. Dart shooting occurs towards the end of this stage.

Stage 6: The penis is maximally everted.

Following dart shooting, the courting snails align themselves for simultaneous intromission. They remain immobile for 2-6 hours while spermatophore exchange takes place (Adamo and Chase, 1988).

## 1.3 Central control of courtship in gastropods

The central control of courtship has been well studied in gastropod mollusks, largely owing to their relatively simple nervous systems, and the possibility of mapping an observable behavior to a particular neural circuit. In this passage, I will give an overview of central control of courtship in three species of snails: The pond snail *Lymnaea stagnalis* (order Basommatophora),

the California sea slug *Aplysia californica* (order Anaspidea), and the common garden snail *Cornu aspersum* (order Stylommatophora).

### 1.3.1 Central Control of courtship in *Lymnaea stagnalis*

*Lymnaea stagnalis* is a simultaneous hermaphrodite whose courtship and mating behavior have been studied in detail. *In vivo* recordings in this species have shown that neurons in the anterior lobe of the right cerebral ganglion are selectively active during different copulatory acts (De Boer *et al*., 1997). A motor function was attributed to this cluster of neurons, since stimulating them, *in vivo*, elicited the eversion of the prepetium, which is one of the stereotypic male copulatory acts. Further evidence for the involvement of the anterior lobe in controlling male copulatory behavior comes from the fact that certain neuropeptides such as APGWamide and conopressin are co-localized in 60% of anterior lobe neurons, and APGWamide containing axons are located in the muscles of the male copulatory organs. It was suggested that these two peptides modulate peristaltic movements in the vas deferens, and thus are important in the transport of semen (Van Golen *et al*., 1995).

### 1.3.2 Central Control of courtship in *Aplysia californica*

*Aplysia californica* is another hermaphrodite with functional male and female reproductive organs. Sex roles in this species vary in each mating, and often animals form chains during mating (Pennings, 1991). Similar to the

findings in *Lymnaea*, immunocytochemical evidence supports the existence of APGWamide in certain populations of central neurons (Fan *et al*., 1997). Furthermore, APGWamide immunoreactive axons are found in the reproductive tract of *Aplysia*, thus supporting the hypothesis that this peptide plays a role in controlling male mating behavior in this species.

### 1.3.3 Central Control of courtship in *Cornu aspersum*

The cerebral ganglion in *Cornu* is divided into the procerebrum, mesocerebrum and postcerebrum (Nabias, 1894). It was initially observed that the right mesocerebrum is larger than the left (Kunze, 1921) but not much was known about the exact differences between the two, or the function of mesocerebral neurons, until the past two decades. Chase (1986) presented evidence indicating that the right mesocerebrum has 23% more neurons than the left mesocerebrum, and that they are 24% larger. In addition to this anatomical evidence, the role of the mesocerebrum in courtship behavior has been established following extensive research involving different methods:

- The extracellular stimulation of the right mesocerebrum (but not the left) resulted in contractions of the dart sac (Chase, 1986). Furthermore, the intracellular stimulation of individual mesocerebral cells caused contractions in the dart sac, penis, or both.
- Mesocerebral neurons reach their maximum size at the same time when the animal is sexually mature (LaBerge and Chase, 1992).

- Live fine wire recordings (Koene *et al*., 1999), where both motor and sensory functions concerning courtship behavior were attributed to mesocerebral neurons.
- Anterograde labeling showed that 96% of mesocerebral neurons have axons in the cerebropedal connective (CPC), 25% of which continue to the dart sac (Li and Chase, 1995).

Further evidence pertaining to the role of the mesocerebrum as a center for integrating mating behavior comes from electrophysiology, where the mesocerebrum is capable of inhibiting the neural network underlying avoidance behavior (Balaban and Chase, 1989). Typically, the avoidance response follows tactile stimulation; however, this response is suppressed during courtship and mating, and even dart receipt fails to trigger the avoidance response when the animal is sexually aroused.

Although courtship behavior varies in each of the aforementioned species, its central control seems to be conserved (Koene *et al*., 2000). The right mesocerebrum of *Cornu aspersum*, the anterior lobe of *Lymnaea stagnalis*, and the H-cluster of *Aplysia californica* have been proposed to be homologous based on immunohistochemical and functional evidence in their role as centers for controlling courtship and mating (Koene *et al*., 2000).

## 1.4 Central pattern generators (CPGs)

Central pattern generators are defined as neural circuits that generate rhythmic patterns of neural activity that drive repetitive body movements (Marder and Calabrese, 1996). This capacity is conserved throughout the animal kingdom (Orlovsky *et al*., 1999). When discussing CPGs, several points have to be taken into account:

- It is important to keep in mind that although CPGs are capable of creating certain rhythmic activities without extrinsic information, neuromodulators might still be required to activate them (Marder and Bucher, 2001).
- The term CPG refers to a function and not to a discrete anatomical identity (Grillner and Wallén, 1985). Indeed, the concept of the CPG being a discrete anatomical unit has long been challenged following evidence that the restructuring of certain CPGs can create, *de novo*, the CPG for a different behavior in lobsters (Meyrand *et al*., 1991).

In the subsequent paragraphs, an overview of CPGs in vertebrate and invertebrate models will be given, with special reference to courtship and copulation.

### 1.4.1 Central pattern generators in vertebrates

The first description of central pattern generators dates back almost a century, when T.G. Brown (1914) proposed that the extension and flexion of

limbs (contractions and relaxation of limb muscles) could be controlled by central circuits that work antagonistically. Since then, our knowledge of vertebrate CPGs has gone a long way owing to lower vertebrate models, such as the lamprey, which is an ideal organism for studying CPGs for locomotion (for example, see Buchanan *et al*., 1989).

Most of the work done in human CPGs deals with locomotion (for example, Mackay-Lyons, 2002; Zehr *et al*., 2007). Despite ethical considerations, this field has prospered because of its implications in rehabilitation following injury (for example, Dietz *et al*., 1995).

The role of CPGs in controlling different aspects of reproduction has been studied in both humans and other mammals. Male ejaculation has attracted the attention of neuroscientists because of its implications in treating infertility. In male rats, for instance, a spinal pattern generator is shown to be responsible for the male ejaculatory pattern (Carro-Juaréz and Rodriguez-Manzo, 2005). Further studies have suggested that a similar spinal pattern generator is both present and functional in the female rat as well (Carro-Juaréz and Rodriguez-Manzo, 2006). Moreover, recent evidence suggesting the existence of a functional neuronal network governing male sexual behavior in the brains of female mice has been found (Kimchi *et al*., 2007).

However, despite the great progress made in the field of vertebrate central pattern generation, the gap between behavior and the function of individual neurons remains open.

### 1.4.2 Central pattern generators in invertebrates

There have been more studies of CPGs in invertebrate species than in vertebrate species (Marder *et al*., 2005). Nonetheless, the emphasis has been on locomotion and feeding, these two being readily observable rhythmic behaviors. The CPG for feeding has been studied in a number of gastropod species, especially in the order Basommatophora. In *Lymnaea*, for instance, a sophisticated model involving six different cell types is proposed with well known firing patterns and synaptic interactions among the different interneurons (see Chase, 2002).

Studying pattern generation in courtship, however, differs from studying pattern generation in other behaviors such as swimming, breathing, or feeding. In the snail, *Cornu aspersum*, courtship consists of a well-described behavioral repertoire. This repertoire can be broken down into several linked units, each dealing with a single muscle or a very small group of muscles. Different neural networks, which may or may not be found in the same ganglion, could be generating those motor outputs. Lind (1976) first proposed the possibility that there is a pattern generator controlling courtship or at least certain aspects of it. External stimuli, in his opinion, only play a regulatory role instead of controlling the behavior. Neuromodulators enable a certain CPG to produce more than one motor pattern by changing the synaptic and cellular properties of the neurons forming the circuit. Neuromodulation has also been recognized as an intrinsic component of some CPGs (Katz, 1998). In *Lymnaea stagnalis*, for instance, copulation is considered to consist of a series of acts under neuromodulatory

control instead of being a fixed action pattern. Variables such as the motivational state of the animal play a role in whether these acts are executed or not (De Boer *et al*., 1997). Whether there exists a CPG for copulation and courtship, and whether the neuromodulators are intrinsic or extrinsic to the CPG circuit remain to be discovered.

So far, we have no evidence that there exists a CPG for courtship in *Cornu*. The central control of copulation, mainly ejaculation, has been well studied in this species (Hutcheson, 2005). Sperm release in this snail is thought to exhibit aspects of both reflex patterns and fixed action patterns, thus arguing against the possibility of the existence of a CPG. This thesis will explore the possibility of the existence of one or more CPGs responsible for governing different aspects of courtship behavior.

## 1.5 Experimental design

Previous studies have clearly shown that the right mesocerebrum is at least partially in control of courtship and mating, however, there are so far no data pertaining to the activity of these mesocerebral cells at different stages of courtship. The fine wire recordings done previously had the advantage of recording activity *in vivo* (Koene *et al*, 1999) , but nonetheless had the shortcoming of recording only from a subpopulation of right mesocerebral cells. Furthermore, the data came from a single snail, since the technique is quite difficult to replicate. Instead, the experiments described in this thesis employ *in vitro* nerve stimulation

techniques, which are simpler to carry out and, thus, easier to replicate. Recording mesocerebral activity extracellularly while stimulating nerves that would be stimulated during normal courtship acts is a reliable method of finding correlations between mesocerebral activity and nerve stimulation.

The nerves that were used for the simultaneous stimulation were selected based on the behavioral acts of courting snails. Below is a list of the nerves, along with the regions that they innervate (Bullock and Horridge, 1965):

- The right (RTN) and left (LTN) tentacular nerves supply the right and left superior tentacles respectively.
- The anterior lip nerve (ALN) supplies the skin between the tentacles, in addition to the musculature dorsal and lateral of the buccal cavity.
- The posterior lip nerve (PLN) supplies musculature ventral and lateral to the buccal cavity.
- The medial lip nerve (MLN) supplies the oral lobes and the smaller tentacles.
- The penis nerve (PN) supplies the penis.
- The right cutaneus pedal nerve no. 2 (RCPN2) supplies the dart sac in addition to the skin around the genital pore extending from the superior tentacle anteriorly to the penis posteriorly.

## 1.6 Thesis objectives

The main objective of this thesis is to examine whether there is a correlation between right mesocerebral activity and the behavioral stage the animal displays before dissection. Chapter 1 has provided a general introduction to courtship and copulation, and their central control in snails. Chapter 2 describes the experimental design and the methods employed. Chapter 3 presents the obtained results. Chapter 4 provides a general discussion of the results in an attempt to answer the following questions:

1. How does mesocerebral activity differ in snails interrupted at different stages of courtship?
2. Do mesocerebral cells at different stages of courtship respond differently to nerve stimulation?
3. Is there a central pattern generator for the different courtship acts in *Cornu aspersum*?
4. Are more mesocerebral neurons recruited as the animal progresses to a later stage of courtship?
5. How are the different stages of sexual arousal represented in the right mesocerebrum?

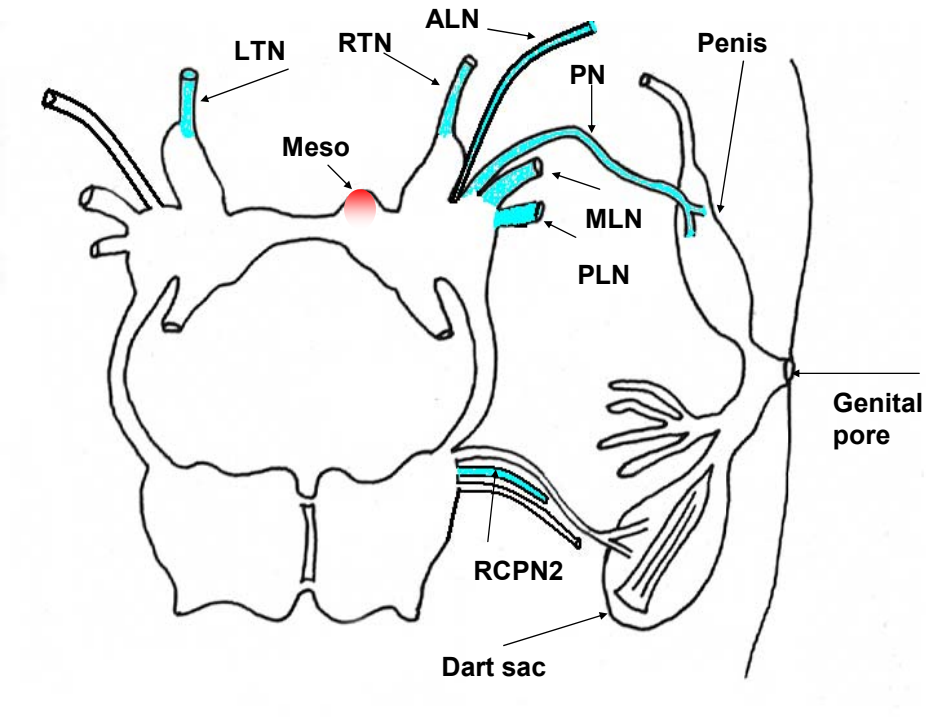



Figure 1.1: Schematic drawing of the central nervous system of the snail, *Cornu aspersum*. The nerves stimulated in the experiments are shown in blue and the right mesocerebrum in red. ALN: Anterior lip Nerve; LTN: left tentacle nerve; MLN: medial lip nerve; PLN: Posterior lip nerve; PN: Penis nerve; RCPN2: Right cutaneus pedal nerve number 2; RTN: Right tentacle nerve, Meso: Mesocerebrum. Adapted from Li and Chase, 1995.

# Chapter 2
# METHODS

## 2.1 Animals

Adult specimens of *Cornu aspersum* (also known as *Cantareus aspersus* and *Helix aspersa*) were imported from natural populations in California. They were maintained in a moisturized container with a 16:8 light: dark cycle at a temperature of 15°C-18°C. Snails were showered every 2-3 days. All animals were sexually mature (curled lip shells) and active, and were fed 50% commercial chicken feed in addition to 50% grain mix. Individual snails were housed in separate compartments (5x5x8 cm) of Lucite boxes to ensure that they only initiated courtship during mating trials.

Courtship behavior has been previously described and divided into distinct behavioral stages by Adamo and Chase (1988). I have modified those stages with the three stages of interest being the following: stage 0, when the animal does not yet show a genital eversion; stage 5, which is characterized by a distinct bulging of the vaginal lobe, and stage 6, when dart shooting occurs (Figure 2.1). In the course of trials, snails were allowed to court their conspecifics until at least one pair reached stage 5 of genital eversion. Trials lasted for 77 minutes on average and animals were dissected after reaching stage 0, 5, or 6 of genital eversion.

## 2.2 Dissections and *in vitro* recordings

Snails were injected with 4-6 ml of isotonic magnesium chloride (67 mM $MgCl_2$, 2.5 mM Tris-HCl) to anaesthetize them prior to dissection. After the removal of the shell, the central nervous system was isolated along with the dart sac, pinned down on a Sylgard-coated dish, and covered with saline. The saline solution had the following composition: 80 mM NaCl, 4 mM KCl, 8 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM Tris at pH 7.8.

The following seven nerves were stimulated: the left tentacle nerve (LTN), the right tentacle nerve (RTN), the anterior lip nerve (ALN), the medial lip nerve (MLN), the posterior lip nerve (PLN), the right pedal cutaneus nerve no. 2 (RPCN2), and the penis nerve (PN). Since it would not be feasible to have eight electrodes in the Faraday cage, the PN, MLN and PLN were sucked into one electrode, and the RTN and ALN into another. The same polyethylene tips were used for all preparations. These particular nerves were selected for nerve stimulation because they have afferent fibers which supply areas that are stimulated during courtship and mating (Bullock and Horridge, 1965).

During the extracellular stimulation of the aforementioned nerves, activity was recorded extracellularly from the right mesocerebrum (Figure 2.2) using a suction polyethylene electrode (diameter =350 μm). The connective tissue covering the ganglion was carefully cleaned and the entire right anterior mesocerebrum was sucked into the electrode using suction provided by a syringe. To minimize the variation in the number of cells being recorded from, the same amount of suction was used across all preparations to draw the right

mesocerebrum into the suction tip by keeping the volume of the saline inside the syringe constant. Moreover, care was taken that the angle of approach of the suction electrode remained comparable across different preparations. This method of extracellular recording is non-invasive and no damage to the mesocerebrum was observed following the recordings.

## 2.3 Experimental protocol

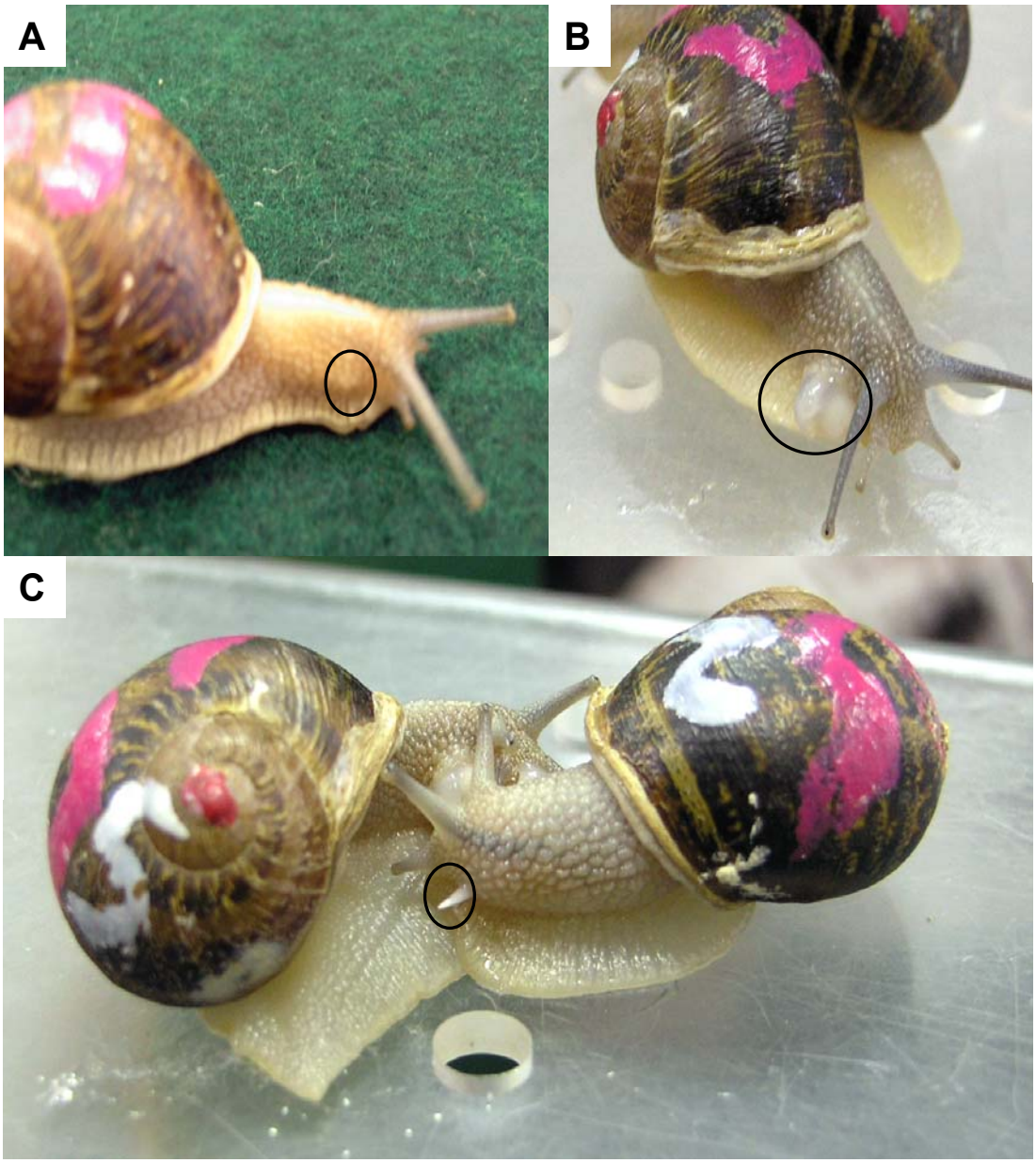
All four stimulating electrodes were connected in parallel to an electronic stimulator (S48, Grass Instruments). Stimulus pulses were isolated from ground (SIU5, Grass instruments). The stimulation protocol (Figure 2.3) was adopted following preliminary studies. To study the effects of different stimulation frequencies and durations on mesocerebral activity, the protocol consisted of four parts: **a**, **b**, **b′**, and **c**. **a** had a low stimulus train frequency of 0.015 Hz, while **b**, **b′** and **c** had a frequency of 0.15Hz. Stimulus trains lasted for 10 minutes in **a**, **b** and **b′** and for 20 minutes in **c**. The seven nerves were simultaneously stimulated with the following parameters: Pulse duration = 2 ms, stimulation rate = 10 Hz, train duration = 2 sec, voltage = 5 V. Prior to each part of the stimulation protocol, mesocerebral activity was recorded for 2 minutes. After the end of the stimulation period, activity was recorded for 8 minutes.

## 2.4 Spike detection and quantification analysis

The signal was amplified using an AC differential amplifier (A-M systems Inc., model 3000), and filtered (high pass 100 Hz, low pass 300 Hz) prior to digitization (Axon instruments, 100 KHz sampling rate). Spikes were detected by threshold recognition and spike rates were quantified using the Dataview software, version 5.2.2 (J.W. Heitler, University of St. Andrew's). Principal component analysis was performed using the Klustawin software, version 4.0 (J.W. Heitler, University of St. Andrew's). Statistical analysis was performed using SPSS software, version 11.0. To compare the spiking rate across the different groups of snails (non-excited, pre dart shooters, and post dart shooters), the non-parametric Kruskal-Wallis test was used because the data were not normally distributed.

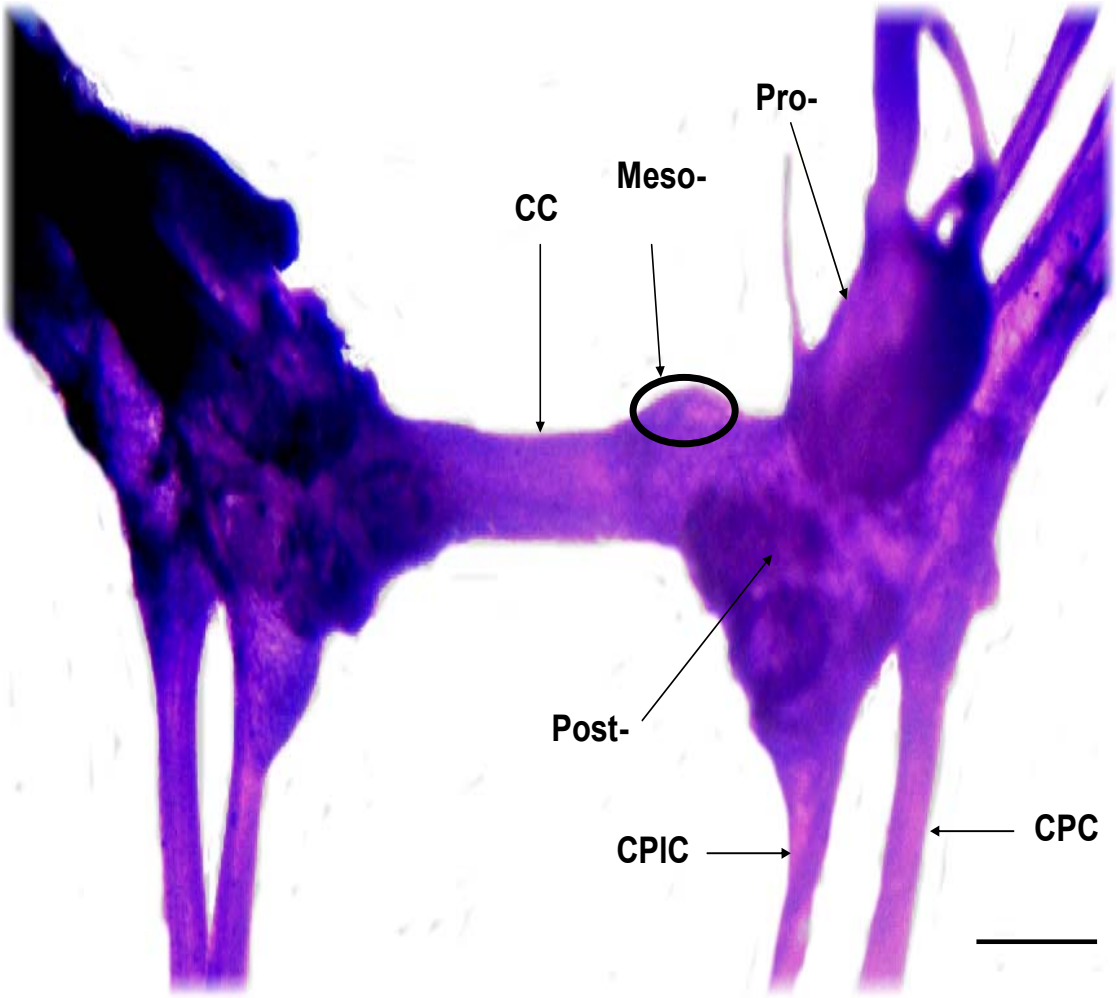
[Figure on next page]

Figure 2.1: Behavioral stages of courtship in the snail, *Cornu aspersum.* **A**: stage 0, with a barely visible genital pore (ellipse). **B**: stage 5: full eversion of both the penial and vaginal lobes (circle). **C**: stage 6: post dart shooting. The animal at the left has shot a dart (ellipse) through the body wall of the animal on the right.


A
B
C

[Figure on next page]

Figure 2.2: Dissected cerebral ganglion of the snail, *Cornu aspersum*. To obtain recordings, the right anterior mesocerebrum (enclosed in the ellipse) was carefully cleaned of connective tissue and sucked into an electrode. The preparation was stained with toluidine blue. Abbreviations: Pro-, procerebrum; Meso-, mesocerebrum; Post-, postcerebrum; CC, cerebral commissure; CPC, cerebropedal connective; CPlC, cerebropleural connective. Scale bar =500μm.


Pro-
Meso-
CC
Post-
CPIC
CPC

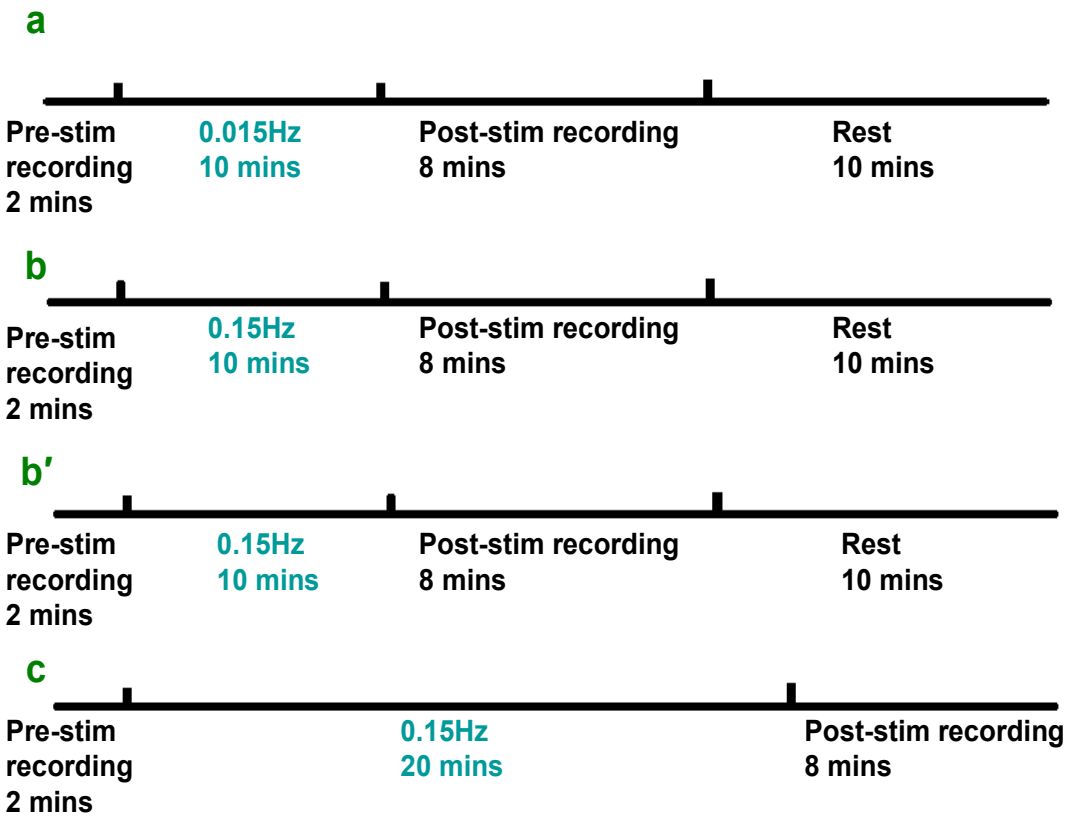



Figure 2.3: The experimental protocol. Mesocerebral activity was recorded 2 minutes prior to each of the stimulations **a**-**c**. The seven nerves were simultaneously stimulated. Stimulus trains had a duration of 10 minutes in **a**, **b** and **b′**, and 20 minutes in **c**. **a** had a stimulus train frequency of 0.015 Hz while **b**, **b′**, and **c** had a frequency of 0.15 Hz. **b′** is a repetition of **b**. Mesocerebral activity was recorded for 8 minutes following the end of the stimulation protocol.

# Chapter 3
# RESULTS

## 3.1 The correlation between baseline mesocerebral activity and stage of sexual arousal

Activity was recorded extracellularly from the entire right anterior mesocerebrum prior to and following the simultaneous stimulation of the seven seven nerves: LTN, RTN, MLN, PLN, RPCN2, PN, and ALN. The four parts of the same protocol (**a**-**c**) were followed for all preparations. Spontaneous mesocerebral activity was recorded for 2 minutes prior to any electrical stimulation in order to establish a baseline rate for the three groups of snails: stage 0 or non excited snails (N=16), stage 5 or pre dart shooters (N=16), and stage 6 or post dart shooters (N=15). In addition, the rate of firing was monitored for 2 minutes prior to **b**, **b′** and **c**. The quantitative results are shown in Figure 3.1. Representative traces of spontaneous activity (prior to stimulation **a**) from the three courtship groups are shown in Figure 3.2.

The non-parametric Kruskal-Wallis test showed that the pre stimulation rate was significantly different (P=0.047) in the three courtship groups, but only prior to **a**. These results show that mesocerebral activity is correlated with the stage of sexual arousal. This is consistent with the results from a fine wire recording *in vivo* where activity increased as the snail progressed from stage 0 to dart shooting (Koene *et al*., 2000). Furthermore, considering that it takes a few minutes after the dissection to prepare each ganglion for electrophysiology, the difference shown here is probably underestimated. In other words, it is probable

that the firing rate was actually higher in pre and post dart shooters prior to dissection than after dissection.

In contrast with the significant difference in firing rates among the three courtship groups prior to **a**, there was no significant difference in firing rate among the three groups before **b**, **b′**, or **c**. This indicates that the stimulation of the seven nerves was sufficient to increase the low firing rate in non-excited snails to a level equivalent to that of pre and post dart shooters, thus suggesting that the stimulation of two or more of the seven nerves *in vitro* mimics the courtship behavior *in vivo*.

Spike detection was done by threshold recognition as described in Methods. However, it can be misleading to set a noise level based on visual inspection alone since there might be spikes that could possibly be considered only noise. To verify that this was not the case, activity was compared before and after treating the preparation with 2-3 ml of 5% glutaraldehyde in 0.1 mol/l sodium cacodylate (pH 7.2). Glutaraldehyde is a neurotoxin and thus no activity should be observed after having administered it. The presumed noise level remained the same before and after the treatment, indicating that it did not contain any action potentials. Furthermore, the qualitative appearance of the waveforms in the presumed noise band remained the same.

## 3.2 Mesocerebral response to nerve stimulation

The effect of the simultaneous stimulation of the seven nerves on mesocerebral activity was examined by monitoring the rate of firing for 8 minutes following each of the stimulations **a-c** (Figure 3.3). On average, **a** failed to increase activity in all three stages, while **b** and **b′** were effective, but only in stimulating non-excited snails (stage 0). **c** increased the firing rate in non-excited snails and in pre dart shooters. None of the stimulations increased the rate of firing in post dart shooter snails. It is important to note here that whenever the stimulation succeeded in raising the firing rate, the rate remained elevated for at least eight minutes.

The foregoing summary incorporates a baseline firing rate that was calculated as the average of all the preparations in a given courtship group. In addition, I also considered changes in firing rates relative to baseline rates that were calculated independently for each experimental preparation. Since the baseline rates had a value <1, considering the ratio between the post stimulation rate and the spontaneous firing rate would result in highly inflated figures. Therefore, here I report the difference between the baseline and post stimulation rates instead of the ratio of the two (Figure 3.4). Taking into account the inter-group variation in spontaneous activity, it is evident that non-excited snails have excitable mesocerebral neurons, since a constant increase is observed after stimulation **a** and through **c**. By contrast, in the case of pre and post dart shooters, even a slight decline in firing rate is seen. The non-parametric Kruskal-Wallis test showed that the difference between the post stimulation and

spontaneous firing rate is significantly different (P=0.03) in the three groups following **c**.

## 3.3 Cluster analysis

The right mesocerebrum has an average of 138 neurons (Chase, 1986). However, most of these seem to be "silent" in unaroused snails since the baseline activity shows that the firing rate is only 0.28spikes/s for stage 0 snails. This rate increases approximately six fold after the completion of the stimulation protocol. In order to determine whether more neurons are recruited after the simultaneous nerve stimulation, template recognition analysis was employed, but it failed to distinguish between different units. This might be due to the fact that the active units have very similar waveforms, or, alternatively, that there are very few units firing. Unfortunately, little is known about the individual waveforms and thus we have no *a priori* basis for favoring one explanation over the other. However, it is viable to approach this problem in a different way by using cluster analysis and comparing the number of clusters before and after nerve stimulation. Although this approach will not help us determine the absolute number of neurons that are active, it will nevertheless give us the relative change in the number of units before and after the stimulation protocol. On this basis, principal component analysis was employed and used to compare cluster number before and after nerve stimulation to establish whether more neurons are recruited following the stimulation protocol, or the same neurons are firing with a higher frequency. My

findings show no support for the former hypothesis since cluster number does not change significantly as the frequency of firing increases (one-sample t-test). Nonetheless, there is a non-significant increase in cluster number in non-excited snails, and a decrease in cluster number in post dart shooters. Figure 3.5 shows the ratios of cluster numbers before and after nerve stimulation in the three groups of snails; however, it does not provide us with any information about the identity of the active units. Further research employing a more sophisticated template recognition software might shed some light on this question.

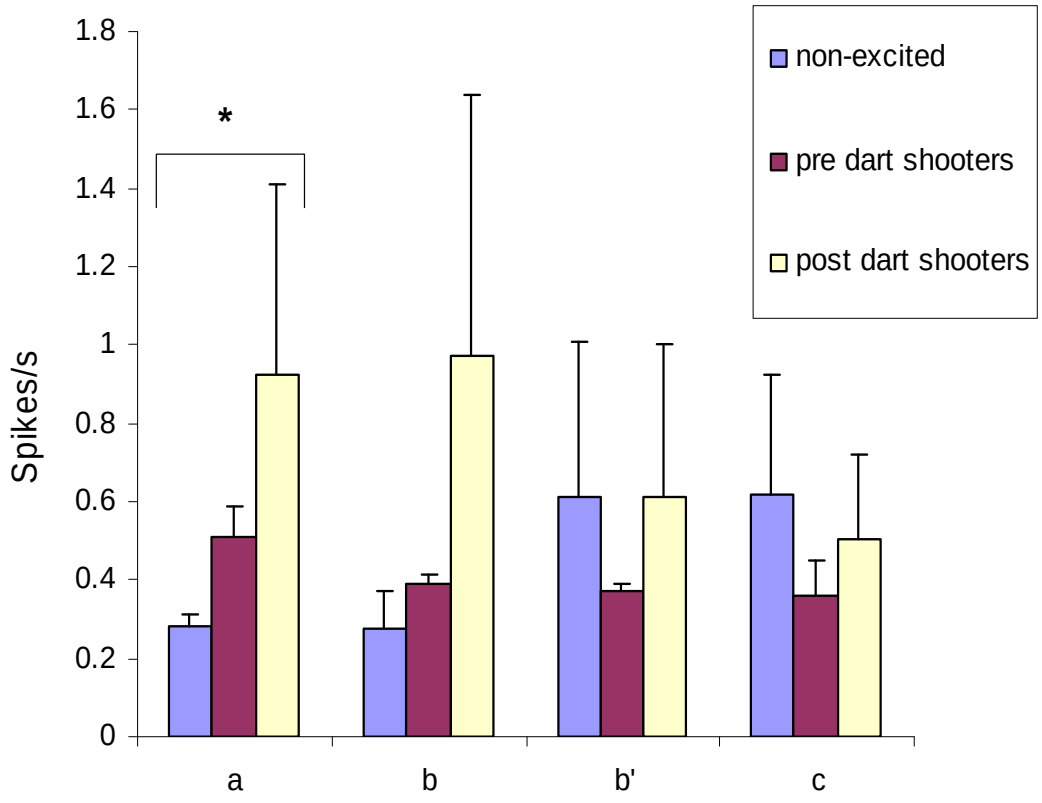



Figure 3.1: Spontaneous mesocerebral activity differs in the three groups of snails. Activity was recorded extracellularly for 2 mins prior to the first stimulus train in **a**, **b**, **b′**, and **c**. Snails belonged to three stages of sexual arousal: Non-excited snails (N=16), pre dart shooters (N=15), and post dart shooters (N=16). Error bars indicate S.E.M. The asterisk indicates a significant difference (P=0.047) in the spontaneous rate of the three groups prior to **a**.

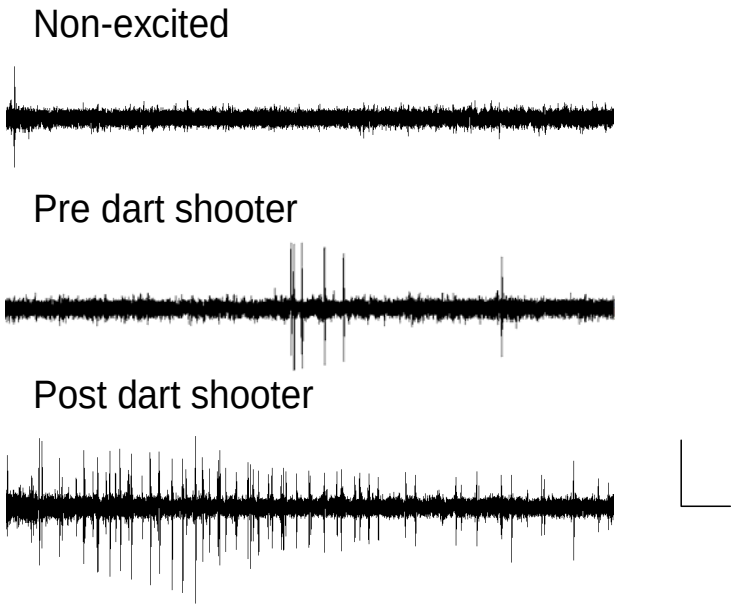
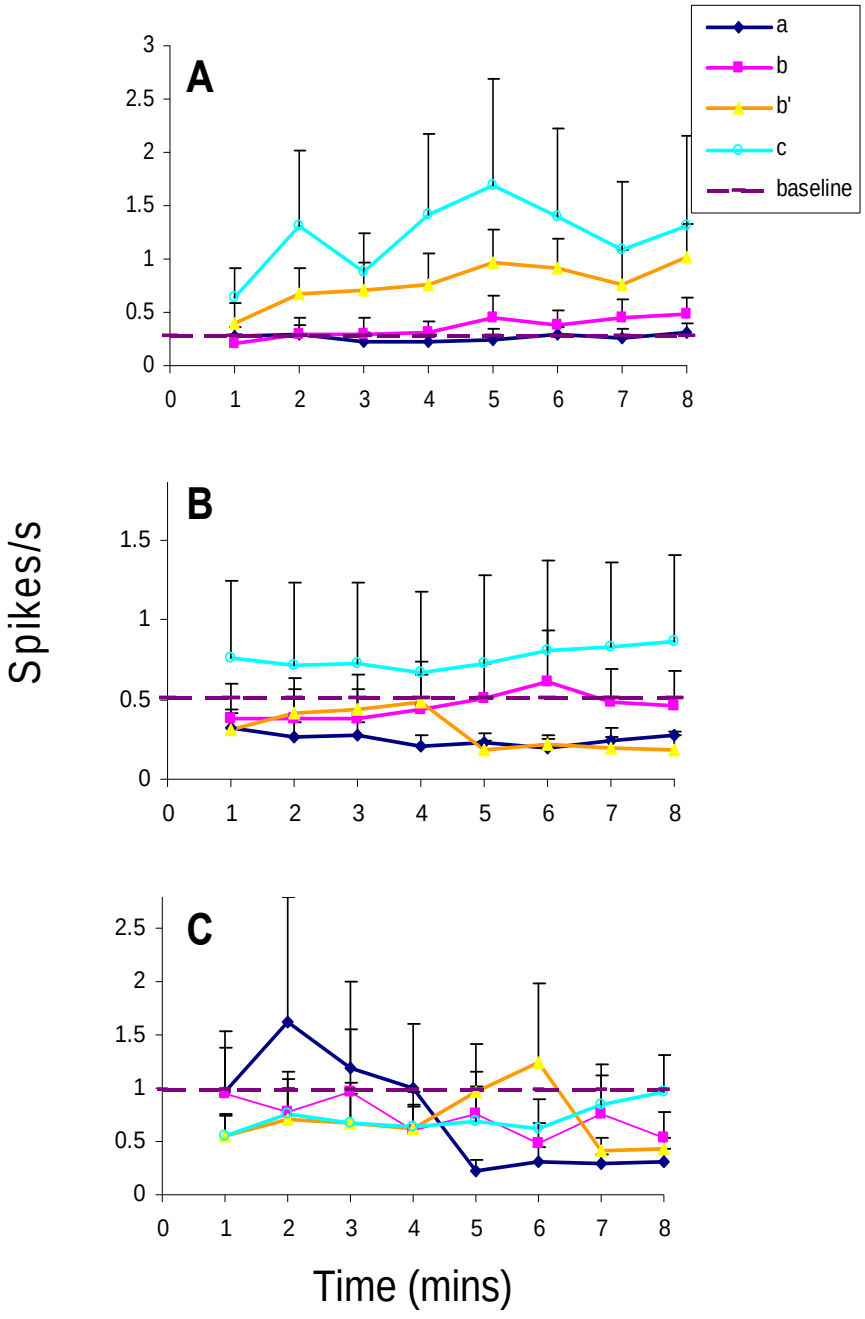



Figure 3.2: Representative traces of baseline activity from animals at each of the three courtship stages. Non-excited snails had the lowest rate of baseline mesocerebral activity, followed by pre dart shooters, and finally post dart shooters. Scale bars = 0.02 mV, 5s.

[Figure on next page]

Figure 3.3: Response of mesocerebral cells to nerve stimulation. Average rate of spiking in: **A**: non-excited snails (N=12), **B**: pre dart shooters, (N=14) and **C**: post dart shooters (N=13) following each of the stimulation protocols **a**-**c**. The dotted lines represent the average pre stimulus **a** baseline rates for each group of snails. Data points represent means±SEM.


A
B
C
a
b
b'
c
baseline
Spikes/s
Time (mins)

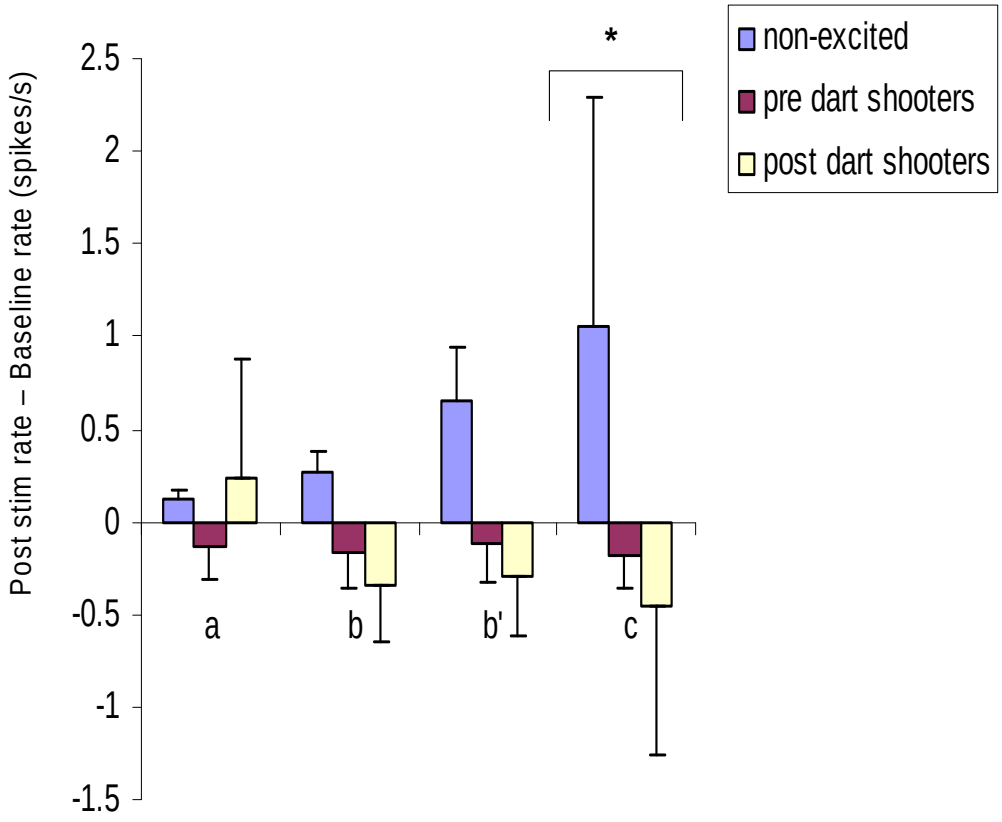



Figure 3.4: The change in the firing rate of mesocerebral cells before and after nerve stimulation. The difference between the average rate of firing 8 minutes following each of the stimulations **a**-**c** and the baseline rate in each preparation. Snails belonged to the following three courtship groups: non-excited snails (N=12), pre dart shooters (N=14), and post dart shooters (N=13). Error bars indicate S.E.M. The asterisk indicates a significant difference (P=0.03) in the three groups after **c**.

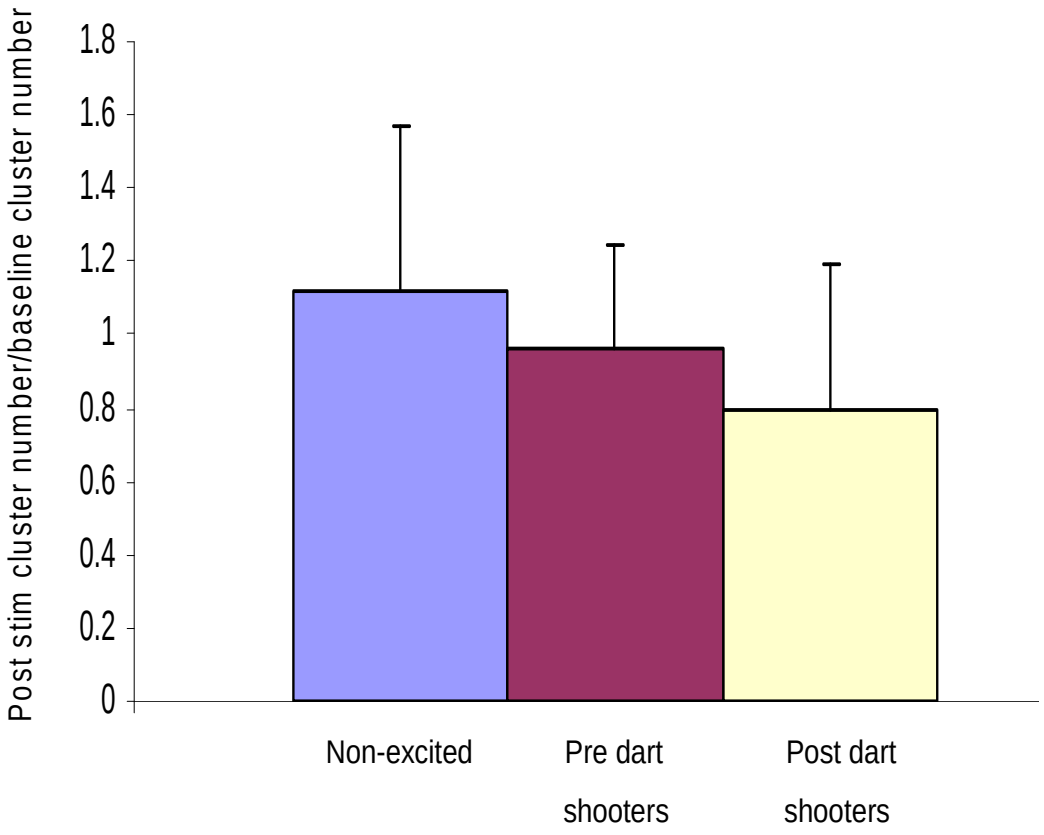



Figure 3.5: No significant change in spike cluster numbers before and after nerve stimulation. Change in cluster number before **a** and after **c** for the three courtship groups: Non-excited snails (N=12), pre dart shooters (N=14), and post dart shooters (N=13). Error bars indicate S.E.M.

# Chapter 4
# DISCUSSION

Previous research using various electrophysiological and immunohistochemical techniques has shown that the right mesocerebrum is involved in controlling courtship in *Cornu aspersum*. In addition, courtship behavior has been studied and documented in detail for this species. However, apart from one *in vivo* recording, we have no data pertaining to mesocerebral activity during different stages of courtship (references in sections 1.2 and 1.3). The results shown in Chapter 3 provide us with an account of mesocerebral activity in animals interrupted at different stages of courtship. They also provide us with a link between behavior and electrophysiology, since the nerves stimulated *in vitro* supply areas that would be stimulated during courtship *in vivo*. So far, we lack a framework for understanding the central control of courtship that is both satisfactorily explanatory and predictive. This thesis serves as a step towards forming such a theory.

## 4.1 Mesocerebral activity correlates with the three stages of sexual arousal

The results outlined in figure 3.1 show a clear correlation between spontaneous mesocerebral activity and stage of genital eversion that the animal displayed prior to dissection. This activity differs significantly in the three groups of snails prior to any electrical stimulation. Previous experiments have shown that mesocerebral

activity is not only correlated to, but a cause of, genital eversion since stimulating the mesocerebrum *in vivo* could excite the snail until the stage of pre dart shooting (Koene *et al*., 2000). The nerves stimulated in the *in vitro* experiments carry both sensory and motor fibers (Bullock and Horridge, 1965) and, as such, the results obtained in the *in vivo* recording are not surprising.

### 4.1.1 Is the right mesocerebrum a central pattern generator for courtship behavior?

Recordings of spontaneous firing rate of mesocerebral cells interrupted at different stages of courtship show that sexually aroused snails have a higher firing rate then unaroused snails. Tinbergen (1951) considered mating behavior to be a chain reaction that is regulated externally. On the other hand, Lind (1976) argued for the possibility of internal programming. One of the simplest ways to see whether there is a central program controlling courtship is to isolate the central nervous from peripheral stimulation. My *in vitro* studies clearly do that and succeed in recording different spontaneous baseline rates in the three groups of snails.

The question, however, remains: Is there a CPG for courtship in the snail? Pattern generators in courtship and reproduction have not been studied previously, mainly because sexual behavior is not a simple cyclic pattern, but involves the complex interaction of various elements (De Boer *et al*., 1997). According to my results, there is at least one neural network in the right

mesocerebrum that is involved in controlling courtship in *Cornu*. The neurons responsible for the motor output involved in courtship can be referred to as CPG neurons, even if not all aspects of the motor pattern found in an intact animals are present. However, the problem with saying that there is a CPG for courtship behavior is that courtship behavior itself is not rhythmic. Central pattern generation deals with neural networks that produce rhythmic patterned outputs (Marder and Calabrese, 1996).Therefore, we can conclude that these neural networks which control courtship in *Cornu* do exhibit some aspects of a CPG, but they are not rhythmic. The problem, then, becomes one of terminology. Hoyle (1970) attempted using the analogy of a "motor tape", where a behavior is executed after an initial command from a certain network. This analogy seems to be very descriptive of the neural control of courtship seen in *Cornu*, and has been used to describe courtship behavior in the grasshopper *Gomphocerus* (Hoyle, 1970). The next step, therefore, would be to try identifying the individual neurons in these non-rhythmic CPG circuits.

Because the mating behavior in snails has intrigued scientists for decades, its neural control has been well studied. Neurons have been found in the right mesocerebrum that can command dart shooting and penial eversion in the snail (Chase, 1986). These command neurons probably are part of the mating CPG in *Cornu*. The term "command neuron" refers to a neuron that can initiate a complex behavior after being stimulated (Wiersma and Ikeda, 1964; Kupfermann and Weiss, 1978). True command neurons are very rare, since not many neurons fulfill the criteria of being necessary and sufficient for

the execution of a certain act. However, based on the fact that mesocerebral activity is correlated with different stages of sexual arousal, there might exist "command-type neurons" in the right mesocerebrum which govern different aspects of the mating behavior.

### 4.1.2 Mesocerebral activity as positive reinforcement

In order to understand the significance of this correlation between mesocerebral activity and behavioral stage, we should first discuss the results of self-stimulation experiments done in *Cornu*. The phenomenon of self-stimulation was first described by Olds and Milner in 1954 and refers to the implantation of an electrode into a certain part of the brain and stimulating that region as reinforcement (positive, negative, or neutral) for a particular behavior. Many scientists have made use of the knowledge that stimulating certain parts of the brain can be rewarding and have used the method of self-stimulation to evaluate the role of those regions in motivation. Self-stimulation in the snail *Cornu aspersum* showed that stimulation delivered to the right mesocerebrum acts as positive reinforcement to an operant response (Balaban and Chase, 1989). Similar results were obtained with the snail *Helix lucorum* (Balaban and Maksimova, 1993). Linking these findings with the fact that the right mesocerebrum controls courtship and mating behavior (Chase, 1986), we can conclude that mesocerebral activity "motivates" the snail to pursue or persist in doing certain behavioral acts that will increase or maintain mesocerebral activity. This could explain why snails are willing to engage in

such a lengthy courtship act. It could also explain why some snails lose interest after getting aroused. Courting snails often engage in circling behavior where they make a full turn or half-turns before reuniting with their partner and resuming courtship (Adamo and Chase, 1988). It was observed that the genital eversion usually decreases during these episodes. During several of my mating trials, some snails lost interest altogether after circling. Therefore, it is possible that if the circling duration exceeds a certain time limit, the snail could lose its genital eversion and would no longer be motivated to mate.

## 4.2 Mesocerebral neurons in non-excited snails respond positively to *in vitro* nerve stimulation

Spontaneous mesocerebral firing rate serves as an indication of the stage of sexual arousal of the animal prior to its dissection. The response to nerve stimulation also differs in the three groups of snails, and serves as another correlate of the behavioral stage, with the only increase in firing rate seen in non-excited snails. It is interesting to note here that the response to the simultaneous stimulation of the seven nerves lasted for at least 18 minutes in non-excited snails. Afterdischarges are thought to be associated with the execution of certain behaviors that are under neuroendocrine control (Kits, 1980). Immunohistochemical evidence from previous research points to an endocrine function for the right mesocerebrum (Chase and Li, 1994), but mesocerebral afterdischarges have never been detected previously in mesocerebral neurons of *Cornu aspersum*. In *Lymnaea stagnalis*, on the other

hand, afterdischarges are readily observed in the anterior lobe (Chase, unpublished), the homologue of the right mesocerebrum in *Cornu*. The long duration of stimulation could be the reason we succeeded in seeing these afterdischarges.

## 4.3 *In vitro* nerve stimulation mimics courtship behavior *in vivo*

Previous evidence has shown that the right mesocerebrum is at least partially in charge of regulating courtship and copulation in the snail. Behavioral observations have provided us with detailed descriptions of the different courtship acts. The research carried out for this thesis bridges the gap between the two. It shows that it is the stimulation of two or more of the seven nerves that causes mesocerebral cells to fire action potentials in non-excited snails. Preliminary studies involving the stimulation of those seven nerves individually failed to induce a significant increase in mesocerebral firing rate. These studies also showed that the input from the RCPN2 was the most influential, followed by the MLN and, finally, the RTN and LTN. These results are in agreement with the behavioral acts of courting snails, where mating partners remain in close proximity to one another, and thus the mesocerebrum needs to receive converging sensory information from different nerves before proceeding to a later stage of courtship.

A possible explanation for the change in the firing rate of mesocerebral neurons is that there is a change in the intrinsic properties of these neurons in snails that are ready to mate. This change can be also under neuroendocrine control, but that was not addressed in this thesis.

## 4.4 The role of the right mesocerebrum in controlling other behaviors

Mesocerebral neurons are active when the animal is sexually aroused. However, these neurons might be multifunctional and thus active in more than one behavior. Such multifunctional "decision" neurons have been described in the leech nervous system, where they are active during both swimming and body shortening (Esch and Kristan, 2002). Another example is an interneuron that was identified in *Aplysia*, and was found to contribute to aspects of at least six different behaviors: locomotion, head turning, defensive head withdrawal, local tentacular withdrawal, rhythmic feeding, and head lifting (Xin *et al*., 1996). Because of the nature of my *in vitro* experimental setup and the use of isolated nervous system preparations, it was not possible to carry out such experiments. However, the quantification of mesocerebral action potentials does suggest that mesocerebral cells are multifunctional. The baseline activity in unaroused snails shows a low average with a high variance (mean rate of 0.28 spikes/s with a standard deviation of 0.65). The fact that mesocerebral activity differs within a group of snails that exhibit the same

stage of genital eversion suggests that mesocerebral activity reflects more than just the stage of sexual arousal of the animal. Gomot (1993) suggested that the mesocerebrum could have a role in controlling spermatogenesis. Another possibility is that the right mesocerebrum could be involved in the control of sperm release. It was shown that the stimulation of the right mesocerebrum could elicit contractions in the distal ovotestis (Hutcheson, 2005). However, no sperm release could be elicited following the electrical stimulation of the mesocerebrum. It could be the case that if mesocerebral activity reaches a certain threshold, it will induce sperm release, or give a permissive signal for it. Alternatively, there could be a combinatorial code and if certain neurons are activated, sperm is released. Another piece of evidence implicating the right mesocerebrum in coordinating egg or sperm release comes from electrophysiological data, where seven out of fifteen right mesocerebral cells were affected by the electrical stimulation of the ovotestis nerve branch of the intestinal nerve (Chase *et al*., 2004).

## 4.5 How is sexual arousal represented in the right mesocerebrum?

The right mesocerebrum of *Cornu aspersum* has an average number of 138 neurons (Chase, 1986). However, most of these seem to be silent in unaroused snails since the baseline activity shows that the firing rate is only 0.28 spikes/s for stage 0 snails. Based on this, right mesocerebral cells may be

described as “dark” neurons. This term was first used by Shoham *et al*. in 2006 and it refers to neurons that fire action potentials rarely and/or to very specific stimuli. According to my data concerning the spontaneous firing rate in unaroused snails, most mesocerebral neurons are “dark”, and a higher firing rate is observed only after the animal progresses towards a later stage of courtship. Principal component analysis was used to compare the number of clusters before and after nerve stimulation to monitor the change in the number of active neurons. Despite the fact that the cluster number does not necessarily refer to the number of neurons, the ratio between the pre stimulation cluster number and the post stimulation cluster number gives us an idea about whether new units are recruited. This ratio did not differ significantly from 1 in all three groups of snails: non-excited, pre dart shooters, and post dart shooters (one-sample t test). Nonetheless, there was a trend showing that the ratio decreased as the stage of sexual arousal increased. The possible recruitment of new units in ganglia from non-excited snails implies that the mesocerebrum does not simply employ a rate code, since the frequency of action potentials is accompanied by an increase in the number of active mesocerebral neurons.

There are two possible explanations for the unchanged number of clusters in pre dart shooters: The same neurons are active before and after nerve stimulation (no change in neuronal activity) or different neurons are active and the mesocerebrum employs a combinatorial code.

In post dart shooters, the number of clusters declines after nerve stimulation, which apparently contradicts the results from the live fine wire recording, where no such decline was seen (Koene *et al*., 2000). Possible explanations for this difference could be that not all seven nerves are active after dart shooting, or, alternatively, the mesocerebrum is no longer in control of the later stages of courtship. The pedal ganglion is an attractive candidate, since it contains a cluster of neurons that is involved in mating (Eberhardt and Wabnitz, 1979), it is the origin of the three cutaneus nerves (Bullock and Horridge, 1965), and it contains the neural network underlying avoidance behavior (Balaban and Chase, 1989). The visceral ganglion is another candidate for a center controlling post dart shooting acts, mainly copulation and sperm release (Hutcheson, 2005).

However, it must be kept in mind that it is not plausible to make a direct comparison between the *in vitro* and *in vivo* results, since the methods employed in distinguishing between the active units were different. Furthermore, the data obtained *in vivo* came from a single animal.

It is important to add here that it is possible that there were new units recruited in all three groups, but were not recordable.

## 4.6 Proclivity versus sexual arousal

Proclivity has been defined as the likelihood of an animal to execute a certain behavior. In the context of courtship, it is the readiness of an animal to

mate when presented with a conspecific. Sexual arousal corresponds to the stage of genital eversion the animal displays at a given time. Previous work has shown that sexual proclivity and sexual arousal are dissociated in the snail, where proclivity is thought to be influenced by isolation and not to require the presence of conspecifics or a particular motor response, whereas sexual arousal does require the presence of a conspecific (Adamo and Chase, 1990). Observations from the mating trials that I carried out suggest that proclivity does not increase with increasing duration of the trial. The percentage of snails showing proclivity remained the same even 350 minutes after the start of the trial. In other words, the willingness to mate is "sensed" by the snail before it even encounters its conspecifics. For unaroused snails, the low firing rate could mean one of two things: the animal is not willing to mate, i.e. the low firing rate is related to the lack of proclivity; or that the mesocerebrum is not active because the animal does not show a genital inversion, i.e. lack of sexual arousal. There is so far no evidence that the former is controlled by mesocerebral neurons. It is likely that proclivity is associated with internal factors such as the availability of autosperm, allosperm, and eggs; and therefore input from the ovotestis via the intestinal nerve should be important. However, there is the possibility of communication between the mesocerebrum and other ganglia to control proclivity as well as sexual arousal. Despite the fact that care was taken to control for confounding variables such as period of isolation, diet, etc, the effects of proclivity and sexual arousal could not be dissociated from one another in my experiments.

A different experimental design is needed to examine the separate effects of proclivity and sexual arousal on mesocerebral activity.

The drive to reproduce is of utmost importance to all species, and is at the root of the different mating strategies and courtship behaviors. This drive is a constant throughout the life of the animal. Proclivity, or the willingness to mate, is a manifestation of this drive. However, it is only shown when conditions are favorable for the animal to reproduce. Availability of gametes, calcium, and various other resources play a role in governing proclivity, to ensure that the animal is ready for a sexual encounter and will benefit from it by reproduction. The central control of proclivity has not been studied yet, but it is possible that the mesocerebrum, together with the visceral and pedal ganglia, plays a role. Sexual arousal is directly related to the stage of genital eversion the animal displays during courtship. Sexual arousal operates on a much narrower timescale and is thought to be controlled by mesocerebral cells (Table 4.1).

## 4.7 Suggestions for future research

One of the questions that remain unanswered is the identity of the units that were active during the extracellular recordings. Unfortunately, template recognition and principal component analysis methods that were used were inadequate in shedding light on this problem. Employing a more sophisticated template recognition software or recording intracellularly from a number of

mesocerebral cells at different stages of sexual arousal might be reasonable alternatives for reaching a conclusive answer.

An important step towards understanding the mechanism of any central pattern generator is to first understand the intrinsic properties of its "building blocks", i.e. the neurons that form it. Neurons that make up CPGs have different dynamics and will respond differently to different synaptic inputs (Marder and Bucher, 2001). Therefore, despite the existence of common features across many CPGs, it is not possible to infer much prior to intracellular recordings. Fortunately, *Cornu aspersum* is an excellent organism for such experiments owing to its relatively simple nervous system and good accessibility using electrophysiological techniques. However, it is equally important to understand that the capabilities of a neural network are more than just the sum of its building blocks, but emerge through the complex interaction between processes at different cellular, synaptic, and network levels (Getting 1989).

The research presented in this thesis focuses on neuronal activity and overlooks the role of neuropeptides and neurotransmitters. Immunohistochemical and electrophysiological evidence point to the involvement of certain neuropeptides in controlling dart shooting and penial eversion. It is hypothesized that FMRFamide is functionally associated with dart shooting and APGWamide with penial eversion (Li and Chase, 1995). Injecting neuropeptides *in vivo*, however, showed that APGWamide evokes genital eversion, and FMRFamide has no effect at all (Koene *et al*., 2000). In

order to establish the function of each neuropeptide, *in vitro* preparations could be helpful. In addition, it would be useful to see their effect on mesocerebral cells at different stages of courtship. It is possible that these neuropeptides act as neuromodulators in the courtship and/or dart shooting CPGs.

Finally, it would be useful to look into the role of other ganglia in controlling courtship and copulation. An important question to answer here is whether there are circuits in other ganglia that are coupled with mesocerebral cells to control courtship. The pedal and visceral ganglia are good candidates, for reasons discussed previously. It would be interesting to record activity from these ganglia and see whether it correlates with behavior and/or mesocerebral activity at the different stages of courtship.

The results presented in this thesis move our knowledge of molluscan reproductive neurobiology a step forward, and I hope they will serve as an inspiration for other researchers to continue the work on gastropod reproductive neurobiology.

Table 4.1: Summary of the hierarchy of drives involved in reproduction.

| | Function | Central control | Pre-requisites | Timescale of operation |
|---|---|---|---|---|
| **REPRODUCTIVE DRIVE** | Ensuring procreation | N/A | Genetic predisposition | Starts at post natal 8-10 weeks and continues throughout most of adulthood |
| **PROCLIVITY** | Prompting the animal to actively seek a mating partner | Possible candidates: right mesocerebrum, pedal and visceral ganglia | Availability of gametes and resources | Days |
| **SEXUAL AROUSAL** | Prompting the animal to carry out courtship and copulation | Right mesocerebrum, possible involvement of other ganglia | Availability of conspecifics | Hours |

# REFERENCES

Adamo S. and Chase R. 1988. Courtship and copulation in the terrestrial snail, *Helix aspersa*. Can. J. Zool., 66:1446-1453.

Adamo S. and Chase R. 1990. Dissociation of sexual arousal and sexual proclivity in the garden snail, *Helix aspersa*. Behav. Neur. Biol., 54:115-130.

Balaban P. and Chase R. 1989. Self-stimulation in snails. Neurosci. research communications., 4:139-146.

Balaban P. and Maksimova O. 1993. Positive and negative brain zones in the snail. Euro. J. Neurosci., 5:768-774.

Brown T.G. 1914. On the nature of the fundamental activity of the nervous centres; together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. J. Physiol., 48:18-46.

Buchanan J.T., Grillner S., Cullheim S., and Risling M. 1989. Identification of excitatory interneurons contributing to generation of locomotion in lamprey: Structure, pharmacology, and function. J. Neurophysiol., 62: 59-69.

Bullock T.H. and Horridge G.A. 1965. Structure and function in the nervous systems of invertebrates. WH Freeman and company. San Francisco.

Carro-Juárez M., Rodríguez-Manzo G. 2005. Evidence for the presence and functioning of the spinal generator for ejaculation in neonatal male rats. Int. J. Impot. Res., 17: 270–276.

Carro-Juárez M. and Rodríguez-Manzo G. 2006. Evidence for the presence of the spinal pattern generator involved in the control of the genital ejaculatory pattern in the female rat. Brain Research, 1084: 54-60.

Charnov E.L. 1979. Simultaneous hermaphroditism and sexual selection. Proc. Natl. Acad. Sci., 76: 2480-2484.

Chase R. 1986. Brain cells that command sexual behavior in the snail *Helix aspersa.* J. Neurobiol., Vol. 17: 669-679.

Chase R. 2002. Behavior and its neural control in gastropod molluscs. Oxford University Press, New York.

Chase R., Antkowiak T., Geoffroy E., and Weatherill D. 2004. Why the ovotestis of *Helix aspersa* is innervated. Acta Biol. Hungarica, 55: 239-249.

Chase R. and Blanchard K.C. 2006. The snail's love-dart delivers mucus to increase paternity. Proc. R. Soc. B, 273: 1471-1475.

Chase R. and Li G. 1994. Mesocerebral neurons and their role in the control of mating behavior. Neth. J. Zool., 44: 212-222.

De Boer P.A.C.M., Ter Maat A., Pieneman A.W., Croll R.P., Kurokawa M., and Jansen R.F. 1997. Functional Role of peptidergic anterior lobe neurons in male sexual behavior of the snail *Lymnaea stagnalis*. J. Neurophysiol., 78: 2823-2833.

Dietz V., Colombo G., Jensen L., and Baumgartner L. 1995. Locomotor capacity of spinal cord in paraplegic patients. Annals Neurol., 37: 574-582.

Eberhardt B. and Wabnitz R.W. 1979. Morphological identification and functional analysis of central neurones innervating the penis retractor muscle of *Helix pomatia*. Comp. Biochem. Physiol., 63A: 599-613.

Esch T. and Kristan W.B. 2002. Decision-making in the leech nervous system. J. Integ. Comp. Biol., 42:716-724.

Fan X., Croll R.P., Wu B., Fang L., Shen Q., Painter S.D., and Nagle G.T. 1997. Molecular cloning of cDNA encoding the neuropeptides of APGWamide and cerebral peptide 1: Localization of APGWamide-like immunoreactivity in the central nervous system and male reproductive organs of *Aplysia*. J. Comp. Neurol., 387: 53-62.

Getting P.A. 1989. Emerging principles governing the operation of neural networks. Ann. Rev. Neurosci., 12 : 185-204.

Goddard C.K. 1962. Function of the penial apparatus of *Helix aspersa Müller*. Austr. J. Biol. Sci., 15: 218-232.

Gomot P. 1993. Studies on the control of spermatogenic DNA synthesis by the mesocerebrum in the snail *Helix aspersa*. Cell. Mol. Neurobiol., 13: 517-527.

Grillner S. and Wallén P. 1985. Central pattern generators for locomotion, with special reference to vertebrates. Ann. Rev. Neurosci., 8:233-261.

Hoyle G. 1970. Cellular mechanisms underlying behavior. Advances in insect Physiol., 7: 349-444.

Hunt S. 1979. The structure and composition of the love dart (gypsobelum) in *Helix pomatia*. Tissue Cell, 11:51-61.

Hutcheson R. 2005. Nervous control of sperm release in the snail, *Cantareus aspersus*. Master's thesis, McGill University.

Katz P.S. 1998. Comparison of extrinsic and intrinsic neuromodulation in two central pattern generator circuits in invertebrates. Exp. Physiol., 83: 281-292.

Kimchi T., Xu J., and Dulac C. 2007. A functional circuit underlying male sexual behavior in the female mouse brain. Nature, 448 :1009-1015.

Kits K.S. 1980. States of excitability in ovulation hormone producing neuroendocrine cells of *Lymnaea stagnalis* (Gastropoda) and their relation to the egg-laying cycle. J. Neurobiol., 11: 397-410.

Koene J. M. and Chase R. 1998. Changes in the reproductive system of the snail *Helix aspersa* caused by mucus from the love dart. J. Exp. Biol., 201:2313-2319.

Koene J.M., Jansen R.F., Ter Maat A., and Chase R. 1999. An *in vivo* electrophysiological study of mating behavior in the snail *Helix aspersa*. Invert. Reprod. Dev., 36:123-127.

Koene J.M., Jansen R.F., Ter Maat A., and Chase R. 2000. A conserved location for the central nervous control of mating behavior in gastropod mollusks: Evidence from a terrestrial snail. J. Exp. Biol., 203: 1071-1080.

Kunze H. 1921. Zur Topographie und Histologie des Centralnervensystems von *Helix pomatia*. L.Z. für Wiss Zool., 118:25-203.

Kupfermann I. and Weiss K. 1978. The command neuron concept. Behav. Brain Sci., 1: 3-39.

LaBerge S. and Chase R. 1992. The development of mesocerebral neurons in the snail *Helix aspersa maxima*. Can. J. Zool., 70:2034-2041.

Leonard J. 1992. The "love-dart" in helicid snails: a gift of calcium or a firm commitment? J. Theor. Biol., 159:513-521.

Li G. and Chase R.1995. Correlation of axon projections and peptide immunoreactivity in mesocerebral neurons of the snail *Helix aspersa*. J. Comp. Neurol., 353:9-17.

Lind H. 1976. Causal and functional organization of the mating behavior sequence in *Helix pomatia* (Pulmonata; Gastropoda). Behav., 59: 162-202.

Mackay-Lyons M. 2002. Central pattern generation of locomotion: A review of the evidence. Phys. Ther., 82:69-83.

Marder E. and Bucher D. 2001. Central pattern generators and the control of rhythmic movements. Curr. Biol., 11: 986-996.

Marder E., Bucher D., Schulz D.J. and Taylor A.L. 2005. Invertebrate central pattern generation moves along. Curr. Biol., 15: 685-699.

Marder E. and Calabrese R.L. 1996. Principles of rhythmic motor pattern generation. Physiol. Rev., 76:687–717.

Meyrand P., Simmers J., and Moulins M. 1991. Construction of a pattern-generating network with neurons of different networks. Nature, 351: 60-63.

Nabias B. de 1894. Rechereches histologiques et organologiques sur les centres nerveux des gasteropodes. Acta Soc. Linn. Bordeaux, 47: 11-202.

Olds J. and Milner P. 1954. Positive reinforcement produced by electrical stimulation of septal area and other regions of the rat brain. J. Comp. Physiol. Psychol., 47:419-427.

Orlovsky G.N., Deliagina T.G. and Grillner S. 1999. Neuronal control of locomotion: from mollusc to man. Oxford University Press, Oxford, UK.

Pennings S.C. 1991. Reproductive behavior of *Aplysia californica* Cooper: diel patterns, sexual roles and mating aggregations. J. Exp. Mar. Biol. Ecol., 149: 249-266.

Shoham S., O'Connor D., and Segev R. 2006. How silent is the brain: Is there a "dark matter" problem in neuroscience? J. Comp. Physiol. A, 192:777-784.

Tinbergen N. 1951. The study of instinct. Clarenden, Oxford.

Tompa A.S. 1984. Land snails (Stylommatophora). In Tompa A.S., Verdonk N.H., and Van Den Biggelaar, J.A.M. (eds.). The Mollusca, vol.7: Reproduction. Acadmic Press, London.

Van Golen F.A., Li K.W., De Lange R.P.G., Van Kesteren R.C., and Geraerts W.P.M. 1995. Co-localized neuropeptides conopressin and Ala-Gly-Trp-NH2 have antagonistic effects on the vas deferens of *Lymnaea*. Neurosci., 69 (4): 1275-1287.

Webb G.R. 1952. Pulmonata: Helminthoglyptidae. Sexological data on the landsnails *Cepolis maynardi* and *Helminthoglyptidae traski fieldi* and their evolutionary significance. Gastropodia 1: 4-5.

Wiersma, C.A.G., and Ikeda K. 1964. Interneurons commanding swimmeret movements in the crayfish, *Procambarus clarkii* (Girard). Comp. Biochem. Physiol., 12:509-525.

Xin Y., Weiss K.R., and Kupfermann I. 1996. An identified interneuron contributes to aspects of six different behaviors in *Aplysia.* J. Neurosci., 16: 5266-5279.

Zehr E.P., Balter J.E., Ferris D.P., Hundza S.R., Loadman P.M. and Stoloff R.H. 2007. Neural regulation of rhythmic arm and leg movement is conserved across human locomotor tasks. J. Physiol., 582: 209 - 227.