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BEHAVIOURAL AND NEUROBIOLOGICAL ASPECTS OF DART SHOOTING IN THE GARDEN SNAIL *HELIX ASPERSA*

by

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A thesis submitted to the Faculty of Graduate Studies and Research
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To Cate, Frans, Koosje and Mieke for their love and unlimited support

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 4

Chapter 6

ABSTRACT

Dart shooting, a bizarre component of mating behaviour seen in several species of terrestrial snails, has bewildered scientists for centuries. The hermaphroditic snail *Helix aspersa* pushes a calcareous "love dart", covered with mucus, through the skin of its partner at the end of an elaborate courtship. I address both why this dart shooting behaviour is performed and how it is controlled by the brain. I find that the dart cannot serve as a nuptial gift of calcium because it is rarely internalised by the recipient and contains only a small amount of calcium. However, most shot darts penetrate the skin and come into contact with the blood. I demonstrate that mucus, produced by the digitiform glands and carried on the dart, causes contractions in the female organs. The contractions suggest that more sperm will reach the sperm storage organ as a result of dart shooting, which is important in sperm competition because snails store sperm from several partners before laying eggs. This introduction of a bioactive substance can be explained by either mate choice or mate manipulation. My findings show that dart shooting is an optional component of mating behaviour, which supports the mate manipulation hypothesis. I conclude that the dart transfers a substance to manipulate the storage of the donated sperm. Many species transfer bioactive substances into conspecifics. I propose the term "allohormones" for such substances to distinguish them from hormones and pheromones.

To investigate how the central nervous system controls dart shooting and other mating behaviours, I used an *in vivo* approach. The right mesocerebrum has been proposed as the control centre for mating behaviour based on *in vitro* findings. I demonstrate, by electrically stimulating and recording from right mesocerebral neurones in the intact animal, that these neurones are involved in dart shooting and penial eversion. I also test the hypothesis that different neuropeptides mediate different mating behaviours, and I find that APGWamide is responsible for genital eversion. From my results, together with data obtained using other gastropods, I conclude that the anteromedial portion of the right cerebral ganglion is an evolutionarily conserved region for the control of mating behaviour.

RÉSUMÉ

Le lancement du dard est une composante insolite du comportement sexuel de certaines espèces d'escargots. *Helix aspersa*, un escargot hermaphrodite, lance une "flèche d'amour" composée de calcium et recouverte de mucus à travers la peau de son partenaire après l'avoir courtisé. Cette thèse traite des raisons de ce comportement et de la manière dont le cerveau le contrôle. Mes résultats démontrent que le dard ne peut être une dot de calcium parce qu'il est rarement incorporé par le récipiendaire et parce qu'il ne contient qu'une petite quantité de calcium. Cependant, la plupart des dards qui sont lancés perforent la peau et entrent en contact avec l'hémolymphe. Je démontre que le mucus qui est produit par les glandes digitiformes et recouvre le dard contient une substance qui affecte les organes reproducteurs. L'effet observé suggère que, lorsque le dard est reçu, plus de sperme est emmagasiné par le récepteur. Cet effet est important pour la compétition du sperme parce que les escargots entreposent le sperme de plusieurs partenaires avant de pondre leurs oeufs. L'introduction d'une substance bioactive par le dard peut être expliquée soit comme un choix de partenaire ou comme une manipulation du partenaire. Mes expériences démontre que le lancement du dard est une composante optionelle du comportement sexuel, supportant l'hypothèse de la manipulation du partenaire. Je conclus que l'escargot se sert de son dard pour transférer une substance dans le partenaire, afin de manipuler l'emmagasinage du sperme donné. Plusieurs espèces transfèrent des substances bioactives à leurs congénères. Je propose d'utiliser le terme "allohormone" pour désigner ces substances, les distinguant des hormones et phéromones.

Pour analyser le rôle du cerveau dans le lancement du dard et les autres composantes de l'accouplement, j'ai utilisé une approche *in vivo*. Suite à des expériences *in vitro*, le mesocerebrum droit avait déjà été proposé comme centre de contrôle du comportement sexuel. Je démontre, en procédent à des stimulations et enregistrements électriques de neurones du mesocerebrum droit dans l'animal intact, que ces neurones sont impliqués dans le lancement du dard et l'éversion du pénis. Je teste aussi l'hypothèse que certains neuropeptides sont impliqués dans différents acpects du comportement sexuel. Je démontre que l'APGWamide est responsable de l'éversion

génitale. Mes résultats, jumelés aux résultats disponibles et obtenus dans d'autres mollusques, mènent à la conclusion que la région antéromédiale du ganglion cérébral droit est une région du cerveau qui a été conservée dans l'évolution pour contrôler le comportement sexuel.

STYLE AND CONTRIBUTIONS OF AUTHORS

As an alternative to the traditional thesis format, a dissertation can consist of a collection of papers that have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following (cited from the "Guidelines for Thesis Preparation" of the Faculty of Graduate Studies and Research):

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>
> As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
>
> In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers."

All experimental work presented in this thesis was performed by Joris M. Koene, under the supervision of Ronald Chase. The research done for Chapter 6 was co-supervised by Andries ter Maat and René F. Jansen from the Vrije Universiteit of Amsterdam. Anton W. Pieneman, also from the Vrije Universiteit, taught me how to implant the fine wire electrodes into snails. René F. Jansen helped with the analysis of the electrophysiological recording.

All the chapters in this thesis are written by Joris M. Koene with editorial help of the co-authors. The published chapters are used with copyright permission of these co-authors and the publishers of the scientific journals. The different formatting styles in Chapters 2 to 6 are due to the different requirement for the scientific journals.

Chapters 1 and 7 have been written exclusively for this thesis.

Chapter 2 has been published with R. Chase in the *Journal of Molluscan Studies* **64**: 75-80 (Oxford University Press).

Chapter 3 has been published with R. Chase in the *Journal of Experimental Biology* **201**: 2313-2319 (Company of Biologists Ltd.).

Chapter 4 has been submitted for publication with R. Chase.

Chapter 5 has been prepared for publication.

Chapter 6 will be published with R.F. Jansen, A. ter Maat and R. Chase in the *Journal of Experimental Biology* (in press). Some of the data have also been published, with the same co-authors, in the Proceedings of the 8th International Congress of Invertebrate Reproduction and Development, *Invertebrate Reproduction and Development* **36**: 123-127 (1999).

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Below is a list of the contributions to original knowledge about the function and neuronal control of dart shooting behaviour in a terrestrial snail.

1. The thesis gives a new explanation for why love darts are shot during the courtship of some terrestrial snail species.
   1.1. The long-standing belief that the dart is a gift of calcium to the mating partner for the production of eggs is overturned (Chapter 2).
   1.2. Instead, it is demonstrated that the mucus carried on the dart has effects on the female reproductive system that can lead to an increase of the number of sperm that reach the sperm storage organ (Chapter 3).
   1.3. Based on a theoretical model and empirical data, it is concluded that the dart is an instrument used for mate manipulation and not for mate choice (Chapter 4).
   1.4. The new term "allohormones" is proposed as a name for bioactive substances that are transferred to conspecifics, and that directly influence the recipient's reproductive physiology (Chapter 5).
2. The presented neurobiological data in Chapter 6 support the previously proposed idea that the anteromedial region of the right cerebral ganglion is an evolutionarily conserved region that controls mating behaviour in gastropods.
   2.1. The *in vivo* recording method for aquatic molluscs is modified to be applied to terrestrial snails. With this method, the first successful multi-neuronal *in vivo* recordings in terrestrial snails are made from which the individual neuronal units can be isolated.
   2.2. The activity of the individual mesocerebral units is correlated with either dart shooting, penial eversion, or both. Most neurones also receive tactile information from a specific skin area.
   2.3. Genital eversion can be evoked by electrical stimulation of the right mesocerebrum. This eversion is mediated by the APGWamide-containing neurones of this brain area, as was previously hypothesised.

ACKNOWLEDGEMENTS

Most acknowledgements in theses begin with thanking the supervisor. Not conforming to this habit, I want to start by thanking Andries ter Maat without whom I might never have ended up doing my Ph.D. research at the McGill University. It was he who first introduced me to Ronald Chase and who suggested I should go and do a research project in his laboratory. I had just finished a research project in Andries ter Maat's laboratory at the Vrije Universiteit in Amsterdam; I decided to take up the challenge. After this research project in the Chase lab, I obtained the Dutch equivalent of a Master's Degree. Having enjoyed working in the laboratory of Ronald Chase, I decided to return there to continue the research on the fascinating dart shooting behaviour of snail.

Of course, I am much indebted to Ronald Chase for his encouragement and guidance throughout my Ph.D. programme. Also, I have greatly appreciated his support and scepticism for my ideas and research plans. It has been a great pleasure working with Ronald Chase as well as all the people in his laboratory: Marie-Ève Fortier, Michael A. Landolfa, Mathieu Lemaire, Steven A. Prescott, Stéphanie Ratté, David Rogers, and Aurelie Shapiro. In particular, I thank Stéphanie Ratté and David Rodgers for their valuable suggestions and comments after reading many parts of this thesis, and Michael A. Landolfa for his input into Chapters 3 and, especially, 4.

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use his computer and for helping me to analyse the electrophysiological recording of Chapter 6. For the experimental methods in Chapter 6, I am indebted to Anton W. Pieneman for giving unlimited technical assistance and for teaching me the *in vivo* techniques. I have valued Pamela A.C.M. de Boer's encouragement and comments to Chapters 4 and 6.

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Chapter 1

THESIS INTRODUCTION

Scientists have been, and are still, intrigued by the amazing secondary sexual characteristics with which some animals are equipped. The evolution of these traits and their associated behaviours can be explained by the theory of sexual selection, first proposed by Darwin in 1871. Sexual selection is based on the differential reproductive success of individuals, and involves processes like mate choice and competition for mates. Contrary to natural selection, extreme features that can hinder survival of the animal are often selected for. Well known examples are the extravagant tail feathers of birds (Møller, 1998), the impressive antlers of ungulates (Clutton-Brock, 1981), and the large nuptial gifts of insects (Proctor *et al.*, 1995).

One behaviour that remains to be explained by the theory of sexual selection is the bizarre dart shooting that is seen in several species of terrestrial snails (Baur, 1998). This courtship behaviour consists of each hermaphrodite mating partner forcefully stabbing a so-called "love dart" through the skin of the mating partner, where it usually stays lodged (Figure 1). Despite the injury, mating partners show only a small avoidance response and continue their mating behaviour.

In this thesis, I attempt to understand both the function of dart shooting and the means by which dart shooting is expressed by the central nervous system. Therefore, my thesis research on the love dart called for a variety of approaches, including behavioural, physiological, game theoretical, and neurophysiological. In the first part of this introductory chapter, I give an overview of mating behaviour and dart shooting in snails with special reference to the studied species, the garden snail *Helix aspersa*. I also discuss the hypotheses that have been proposed to explain dart shooting behaviour, and I formulate the first central question dealt with in Chapters 2, 3, and 4: What is the function of dart shooting? In the second part of this chapter, I focus on the neurobiological aspects of dart shooting and mating behaviour in snails, and I formulate the second central question dealt with in Chapter 6: How are dart shooting and the other components of mating behaviour controlled by the central nervous system?

Behavioural aspects of dart shooting and mating

Courtship and copulation in snails

Dart shooting behaviour constitutes only part of an elaborate mating sequence, therefore, I shall also include other components of the mating behaviour. Dart shooting is exclusive to the Stylommatophora, an order of pulmonate land snails characterised by simultaneous hermaphroditism. In such hermaphrodites each mating partner fulfils both the male role and the female role during a given mating encounter. Adamo and Chase (1988) described the mating behaviour of *Helix aspersa* in detail. This snail's mating behaviour consists of three phases: introduction, dart shooting, and copulation. At the start of introduction two potential mating partners meet and initiate tentacle contacts and lip contacts. During these behaviours, the genital atrium - which is normally internal - is slowly everted through the genital pore and becomes visible as a white bulge on the right side of the head. This is referred to as the genital eversion and exposes the female and male genital openings, but leaves the penis inside the animal. The animals position themselves head-on and occasionally interrupt courtship to crawl in a circle or several semicircles to reposition themselves with regard to the partner (circling behaviour: Adamo & Chase, 1988). In the advanced stages of genital eversion, which are numbered 1 to 5 based on their form and increasing size (Adamo & Chase, 1988), animals will often make lip-genital contacts and occasionally bite each other. When the higher levels of sexual excitement are reached the animals often push their genital eversions together. These behaviours are performed during half an hour on average, although they can continue for up to one hour (Adamo & Chase, 1988).

The introductory phase ends when one of the mating partners shoots its love dart. The term "love dart" is used because of the analogy with Cupid's arrow (reviewed by Kothbauer, 1988). The dart, however, does not actually fly through the air as the term "dart shooting" implies. Instead, it is expelled by a forceful eversion of the muscular dart sac, from the genital eversion, when the mating partners are in close bodily contact (Adamo & Chase, 1988). Normally, the dart detaches from the dart sac when it pierces through the partner's skin (Figure 1), therefore, it remains lodged in the recipient and can be internalised into the body cavity (Adamo & Chase, 1990). Usually, not long after one

individual of the mating pair has shot its dart, the partner will do the same (average interval eight minutes; Adamo & Chase, 1988).

Once a snail has shot its dart it will attempt to copulate by everting its penis through the male pore of the genital eversion (stage 6 eversion: Adamo & Chase, 1988) and try to intromit into the female genital opening of the partner. Successful copulation may take multiple attempts because the penes of both animal have to be inserted simultaneously, and an individual will not allow penis intromission until it has reached the stage of penis eversion (Chung, 1987). Once simultaneous intromission is achieved the snails become inactive (Figure 1), partly withdraw their tentacles, and remain in this copulatory position for several hours (average 422 min; Adamo & Chase, 1988). During this period spermatophores are formed and transferred, one by each mating partner.

An overview of the morphological structures described below is given in Figure 1 of Chapter 3 (page 33). The spermatophore is formed in the penial complex and consists of four parts: The head, the neck, the body and the tail. The empty head and neck of the spermatophore are probably formed in the penis; the body of the spermatophore is formed in the epiphallus and envelopes the sperm; the tail of the spermatophore is produced by the flagellum (Lind, 1973). After formation, the spermatophore is transferred into the bursa tract diverticulum of the partner. This organ forms part of the bursa complex, which consists of the bursa tract diverticulum and the bursa copulatrix with its accompanying bursa tract. Sperm swim through the tail of the spermatophore to reach the spermoviduct via the genital atrium (Lind, 1973). Only a small percentage (0.1 % in *Helix pomatia*: Lind, 1973) eventually reach the sperm storage organ, the spermatheca, at the end of the spermoviduct. The vast majority of the sperm are instead transported through muscular actions - together with the spermatophore - to the bursa copulatrix where they are digested (Lind, 1973). For *Helix pomatia* it has been shown that digestive secretions from the bursa copulatrix enter the spermatophore after copulation (Hryniewiecka-Szyfter & Redziniak, 1976). However, in that species the spermatophore is received directly in the bursa tract. Whether such digestive enzymes also enter the spermatophore in the bursa tract diverticulum of *Helix aspersa* is not known.

The sperm that escape digestion and reach the spermatheca can be stored in that organ for over a year before being used for the fertilisation of eggs (Tompa, 1984). The

storage of received sperm is necessary because egg laying does not immediately follow copulation. Moreover, several matings with different partners can take place before eggs are fertilised and laid. The eggs of *Helix aspersa*, as those of many terrestrial snail species, are provided with calcium in the shell (Tompa, 1984). In *Helix aspersa* the average egg clutch contains 50 to 100 eggs (Daguzan, 1981). Hence, a large amount of calcium is required for egg laying (Tompa & Wilbur, 1977).

The love dart of *Helix aspersa*

The nine-millimetre long love dart (see Figure 2) is produced and stored in a special organ, the dart sac (Hunt, 1979). The production or regeneration of a dart takes approximately six days (Tompa, 1982), and production seems to be initiated only after eversion of the dart sac during the first mating (Chung, 1986a). The initiation of dart formation might be triggered by the loss of the gelatinous substance present in the dart sac of virgins (Chung 1986a). The dart is composed almost completely of calcium carbonate ($CaCO_3$) in the form of micro-crystalline aragonite (*Helix pomatia*: Hunt, 1979; *Helix aspersa*: Dillaman, 1981). The calcareous structure is protected by a protein sheath - probably also produced by the dart sac - which represents about 5 to 6 % of the total weight (Hunt, 1979). The dart sac is directly connected to the digitiform glands that secrete a mucus onto the dart immediately before it is shot (Chung, 1986b; Adamo & Chase, 1990).

The shape of the dart is species-specific and is often used by malacologists to distinguish between closely related species (Tompa, 1984). Therefore, Webb (1951) proposed that darts might be involved in species recognition, but this idea found little support. The dart of *Helix aspersa* (Figure 2) consists of a hollow shaft with four blades that are positioned perpendicular to the shaft. Hence, in cross section the dart forms a symmetrical structure resembling a Celtic cross (Hunt, 1979). The shaft is slightly curved and ends in a fine, sharp tip. The blades run along most of the length of the dart, and only disappear near the basal end. This end is called the flare and continues in the corona, which attaches the whole structure to the tubercle at the posterior end of the dart sac. This attachment is usually broken during dart shooting leaving the dart lodged in its target.

Why do snails shoot darts?

The bizarre phenomenon of dart shooting has still not been satisfyingly explained although scientists have pondered on it since the 17th century, when Swammerdam described the dart as an "alkaline little bone"; he suspected that it had something to do with mating (Swammerdam, 1737). Near the end of the 17th century the term love dart ("flèche d'amour") came into use (reviewed by Kothbauer, 1988). More than a hundred years later, when Ashford (1883) wrote his review of British dart shooting species, little more was known about the function of darts. Ashford expressed the general consensus of the time that the puncture caused by the dart somehow excites the mating partner.

The hypothesis that sexual excitement is increased by the mechanical stimulation of the dart was first tested by Goddard (1962). He reported that pinching the skin near the genital pore increased the tonus of the penial muscles. Both Jeppesen (1976) and Lind (1976), however, were unable to confirm Goddard's findings based on observations of the mating behaviour of *Helix pomatia*. Instead, Jeppesen suggested that the dart might mechanically stimulate the recipient's female opening and Lind speculated that the dart serves to test the level of sexual excitement of the partner. Giusti and Lepri (1980) came to the same conclusion as Lind for several related snail species. If the dart serves to increase or test the excitement of the partner, it is expected to be used during every mating encounter. Inconsistent with this idea is the fact that the dart is deciduous and can be used once every six days (the dart regeneration time), while mating occurs more frequently (Tompa, 1984).

Webb (1952) suggested that the mechanical stimulation associated with dart shooting serves to coerce the partner to co-operate in the mating process. Elaborating upon this hypothesis, Leonard (1992) proposed that the dart's purpose is to induce the partner to donate sperm. She based her argument on a sperm-trading model that she had developed earlier (Leonard, 1990), in which she assumed that the female role is preferred in internally fertilising species because of the higher assurance of reproductive success associated with this role. This preference leads to a conflict because each individual will only want to assume the preferred role (Leonard, 1990). Leonard argued that reciprocal mating - i.e. each individual fulfils the male and female role with the same partner either simultaneously or sequentially - has evolved as a solution to this conflict (1990). She

suggested that the dart would have evolved to indicate the honest intention of the shooter to fulfil the less preferred male role, which would encourage the partner to reciprocate (Leonard, 1992). In the application of her model to dart shooting, however, she fails to take into account that dart shooting snails are *simultaneously* reciprocal hermaphrodites, i.e. they mate in both the male and the female role at the same time. Because, these snails donate *and* receive sperm in every mating encounter, there seems no need to stimulate the partner to fulfil its male role, which suggests that neither sexual role is more advantageous. Greeff and Michiels (1999) argued that this should be the case because in hermaphrodites both sexual roles necessarily have, on average, equal reproductive successes.

The hypothesis that the dart could be a gift of calcium was first proposed by Charnov (1979), drawing on insect biology. In insects the donation of valuable nutrients along with the sperm is commonplace. For example, the male arctiid moth *Utetheisa ornatrix* donates an alkaloid - contained in the spermatophore - to the female that protects her, as well as the eggs she supplies with it, against predators (Dussourd *et al.*, 1991). Likewise, the male notodontid moth *Gluphisia septentrionis* transfers a large amount of sodium to the female. This sodium is put into the eggs as a supplement to the sodium-poor diet of the larvae (Smedley & Eisner, 1996). Similarly, in snails the dart could be a nuptial gift of calcium because it is made almost entirely of this element (Hunt, 1979; Dillaman, 1981). Calcium is an important element for growth (Crowell, 1973) and egg laying (Tompa & Wilbur, 1977) in snails. Along the same lines, egg laying could be induced through the mechanical stimulation caused by the dart (Tompa, 1980), or even by the calcium of the dart. Tompa and Wilbur (1977) have reported that the calcium level in the blood shows a large increase right before egg laying. It is conceivable that this increase of calcium in the blood is triggered by the calcium of the dissolving dart, thus evoking egg laying. The calcium hypothesis remains a possible explanation for dart shooting, but it has never been tested.

While the above suggests that the dart might be a gift of calcium, another set of hypotheses is based on the idea that the dart serves as a vehicle to introduce a bioactive substance into the recipient. The dart is covered with mucus from the digitiform glands. In 1925, by injecting digitiform gland mucus into snails, Dorello observed that it caused

the blood to coagulate around the injured skin area. He also found that it contracted the skin and the penis, and reported that it seemed to bring the animal into a state of excitement. Dorello referred to this active substance as *digitana* because it is produced in the digitiform glands. Unfortunately, Dorello's injection experiments did not control for the mechanical stimulus of piercing the skin. Nonetheless, Börnchen (1967) supported the idea that the activity of internal organs could be altered by the introduction of mucus in the snail's haemolymph. Börnchen specifically looked at the effect of the digitiform gland mucus on the isolated heart. He observed an increase in the beating frequency and interpreted this finding as a confirmation of the increase in the overall sexual excitement as reported by Dorello (1925).

The hypothesis that the dart introduces mucus into the recipient and increases excitement has been further tested by Chung (1986b) and Adamo and Chase (1990). In these studies it was found that when extracts of the digitiform glands were introduced into the haemolymph of snails the level of genital eversion was increased. Both studies controlled for the mechanical stimulation of the injection. Adamo and Chase (1990) also confirmed that the mucus on the dart enters the haemolymph of the recipient during natural dart shootings. However, the "pheromonal" effect on sexual arousal was relatively small. Mucus injection decreased mating time by only approximately 6.7 %, affected only animals with a stage 2 or 3 genital eversion, and was only marginally significant ($P = 0.052$, $N = 17$: Adamo & Chase, 1990). Adamo and Chase argued that a decrease in mating time might be significant in making the snails less vulnerable to predation (1988), although Pollard's field study on *Helix pomatia* (1975) indicates that snails are not more likely to be predated upon when they are mating.

In a later publication, Adamo and Chase (1996) suggested - based on their own finding and previously proposed hypotheses (Charnov, 1979; Tompa, 1980; Chung, 1987) - that the dart might be used to either influence the use of the donated sperm or trigger oviposition. This hypothesis is also based upon the introduction of a biochemical substance by the dart. Hormonal factors in the dart's mucus, possibly the same hormones that are normally used to control the snail's internal processes related to reproduction (Adamo & Chase, 1996), would directly influence the partner's reproductive system. Along the same lines, Chung (1987) suggested - based on insect biology - that the dart

could reduce subsequent mating by the recipient, suppress allosperm digestion, displace previously stored sperm, or prevent subsequent sperm storage. In all these cases, the dart would have a function in *intrasexual* selection: the competition among individuals for access to, and fertilisation of, mates.

Adamo and Chase (1996) favoured the idea that the dart serves as a male instrument to manipulate the female function. However, they did not rule out the possibility that the dart evolved due to *intersexual* selection: the choices made by individuals, based on certain traits, for mating partners. In this hypothesis, if snails choose their partners based on dart shooting, the preference for dart shooters could cause a Fisherian runaway process. Fisher's runaway selection theory (1930) assumes that a preference exists for an arbitrary trait, and that therefore such a preferred trait confers a reproductive advantage. The result is a runaway process in which the preferred trait (dart shooting) and the preference become exaggerated. This process can lead to the development of extreme traits as the famous example of the peacock's tail. Alternatively, Charnov (1979) had suggested that dart shooting could be a demonstration of an individual's ability to metabolise and use calcium. Such a sexual signal would also have evolved through intersexual selection, and could be explained with the "good genes" hypothesis (Andersson, 1994), also known as the "handicap principle" (Zahavi, 1975). For such a sexual signal to evolve the trait (dart shooting) has to correlate with the animal's physiological (e.g. calcium) condition (Zahavi, 1975). Only individuals able to show or develop the trait demonstrate their superior quality and will be chosen as mates.

In summary, three hypotheses remain able to provide an adaptive explanation for the phenomenon of dart shooting. First, the calcareous dart could function as a nuptial gift to the mating partner for the investment in eggs: the nuptial gift hypothesis (Charnov, 1979). Second, the dart could transfer a bioactive substance into the blood of the mating partner where it causes some effect on the reproductive system that enhances the shooter's reproductive success: the mate manipulation hypothesis (Adamo & Chase, 1996). Third, the dart could indicate the shooter's quality and serve as a sexual signal for the mating partner: the mate choice hypothesis (Charnov, 1979). I shall deal with these three hypotheses in Chapters 2, 3, and 4, respectively.

Neurobiological aspects of dart shooting and mating

The central nervous system of *Helix aspersa*

The garden snail, as many other molluscs, has a relatively simple nervous system with large, individually identifiable neurones that control a limited behavioural repertoire. These unique features greatly facilitate studying how the central nervous system works, and molluscs have therefore become a model system for many neurobiologists interested in understanding the basic principles of the brain (Kandel, 1976).

The central nervous system of the garden snail (Figure 3) consists of several ganglia that communicate though connectives. The bilateral cerebral ganglia are located above the oesophagus, while the remaining ganglia are suboesophageal. The nerves that connect the cerebral ganglia to the pleural and pedal ganglia of the suboesophageal ganglia ring are called, respectively, the cerebro-pleural and cerebro-pedal connectives. The pleural and pedal ganglia connect to the parietal ganglia, which in turn connect to the visceral ganglion that closes the suboesophageal ganglia ring. The cerebral ganglia are interconnected through the cerebral commissure, thus forming, together with the suboesophageal ganglia, a ring around the oesophagus.

The cerebral ganglion is divided into three parts: the mesocerebrum, the procerebrum and the metacerebrum (also known as the postcerebrum). No special function has yet been attributed to the metacerebrum; in the procerebrum most of the olfactory information from the tentacle nerves is received and processed (Ratté & Chase, 1997); the mesocerebrum is thought to be the central control region of sexual behaviour (Chase, 1986).

Central control of dart shooting and mating

The mesocerebral neurones project to the penis and the dart sac via, respectively, the *nervus penis* and the *nervus cutaneus pedalis primus dexter* (Chase & Li, 1994). The male reproductive organs and the dart sac are located in the right side of the body cavity of the snail. As a result, the mesocerebrum is significantly larger in the right cerebral ganglion. Compared to the left, the right mesocerebral neurones are 23% more numerous, with 138 neurones on average; they are also 24% larger, with an average diameter of 76.8 μm (Chase, 1986). Evidence for the function of the mesocerebrum in mating behaviour

comes from *in vitro* electrophysiological experiments. Chase (1986) reported that when individual mesocerebral neurones were intracellularly stimulated, in semi-intact preparations, muscular responses were evoked in the penis, the dart sac, or both. Hence, these data suggested that the mesocerebrum controls both penial eversion and dart shooting.

The mesocerebrum of *Helix aspersa* has a morphological homologue in the pond snail *Lymnaea stagnalis* that is called the anterior lobe. This structure is located in the same anteromedial region of the cerebral ganglion; it shows a similar bilateral asymmetry; and its neurones also innervate the penis (*Lymnaea stagnalis* does not possess a dart sac). The right anterior lobe neurones cause eversion of part of the penial complex when they are electrically stimulated *in vivo*, i.e. in the intact animal (De Boer *et al.*, 1997). The neuropeptide APGWamide (Ala-Pro-Gly-Trp-NH_2) mediates this eversion, probably by relaxing the penial musculature (De Boer *et al.*, 1997), and this neuropeptide is present in most of the anterior lobe neurones (Croll & Van Minnen, 1992). These data suggest that the anterior lobe of *Lymnaea stagnalis* and the mesocerebrum of *Helix aspersa* are morphologically and functionally homologous (Chase & Li, 1994).

To investigate whether the mesocerebrum also contains APGWamide, Li and Chase (1995) performed an immunocytochemical study on *Helix aspersa*. They found that some mesocerebral neurones contained APGWamide. Most of these neurones had projections into the nerve innervating the penis. Therefore, Li and Chase (1995) suggested that APGWamide-containing neurones mediate penial eversion, as in *Lymnaea stagnalis*. In the same study, other mesocerebral neurones were found to contain FMRFamide (Phe-Met-Arg-Phe-NH_2). Most of these neurones projected into the nerve innervating the dart sac (Li & Chase, 1995). Because the Stylommatophora are the only dart shooting molluscs, and seemed to be the only order of Gastropoda to contain FMRFamide in this region of the brain, Li and Chase (1995) proposed that dart shooting evolved accompanied by a neural system using FMRFamide to control it. The FMRFamide-containing neurones were thus thought to mediate dart shooting. Still other neurones were found to contain both APGWamide and FMRFamide (Li & Chase, 1995). These neurones were believed to represent the ones with projections into each of the nerves innervating the penis and the dart sac, and were thought to be involved in both

components of the mating behaviour, as was also previously suggested by Chase (1986) based on his electrophysiological findings. To test the proposed involvement of the mesocerebral neurones in mating behaviour I use an *in vivo* approach (Chapter 6) that allows me to selectively stimulate and record from these neurones in the intact animal.

The *in vivo* approach

One of the major goals of neurobiology is to understand how animal behaviour is produced and modulated by the central nervous system. Such research involves the identification of the neurones in the central nervous system that are responsible for the expression of the behaviour of interest. The relatively simple molluscan nervous system has a limited number of large, individually identifiable neurones. One can take advantage of such unique features to study the central control of behaviours.

The involvement of neurones in relation to certain physiological processes or behaviours can be inferred from studies of the isolated (i.e. *in vitro*) central nervous system that use intracellular and extracellular electrophysiological techniques. Research on the central nervous system *in vitro* has resulted in knowledge about the activity of neurones, their interactions with other elements of the nervous system, their connections to different organs, and their function. One example is the central role of the neurone C3 in eliciting and co-ordinating the tentacle withdrawal reflex of *Helix aspersa* (Prescott *et al.*, 1997). The exact actions of these identified neurones in the intact animal, however, are often unknown and might be different from what is observed *in vitro*. *In vitro* preparations often require that at least part of the central nervous system is denervated, meaning that some of the incoming information from the periphery, via the nerves, is removed. An important technique that helps to overcome this problem is *in vivo* recording (Parsons *et al.*, 1983). This technique makes it possible to selectively stimulate and record from neurones in the central nervous system of an intact animal. Therefore, this technique provides a way to directly test the predicted functions of neurones - assessed from *in vitro* preparations - by working with the central nervous system in its natural environment. The great advantage of *in vivo* preparations is that features of the nervous system's environment that may be of importance, but that are not present in the *in vitro* preparation, are conserved. This technical advancement has led to results that can link complex behaviours with identified individual neurones. Other techniques make it

possible to identify the neuropeptides that are present in and released by neurones (e.g. Price & Greenberg, 1977), which enables one to demonstrate that the identified neuropeptides mediate the expression of the behaviour linked with these neurones. One example is the involvement of the cerebral giant cell in the feeding behaviour of *Lymnaea stagnalis*, with serotonin as a neurotransmitter (Yeoman *et al.*, 1994).

The unique features of the molluscan central nervous system have led to research focussing on individual neurones and attempting to understand their actions in relation to certain behaviours. However, to control complex behaviours, as mating or egg laying, more than a single neurone is required. In these cases, rather than focussing on single identified neurones, clusters of neurones working together must be identified.

Previous *in vivo* work

The egg laying behaviour of two molluscs, the pond snail *Lymnaea stagnalis* and the sea slug *Aplysia californica*, has been studied very successfully with the above mentioned methods. Therefore, I shall briefly review some of the research done on this behaviour with the purpose of illustrating how an *in vivo* approach can be used effectively in neuroethological studies.

Based on *in vitro* results, egg laying behaviour was thought to be controlled by a bilateral group of neurones in the cerebral ganglia in *Lymnaea stagnalis*, the caudo-dorsal cells. These neurones are electrically coupled and show synchronous bursting activity *in vitro* (De Vlieger *et al.*, 1980) during which they release egg laying hormone (Geraerts *et al.*, 1985). In *Aplysia californica* a bilateral group of neurones in the viceral-parietal (=abdominal) ganglia with similar discharge properties also releases egg laying hormone (Kupfermann, 1967). The only evidence for the existence of a centre that controls egg laying in *Helix aspersa* comes from immunocytochemical work, which suggests that it might be located in the visceral or right parietal ganglion (Van Minnen *et al.*, 1992).

In vivo recordings and stimulations have confirmed that the caudo-dorsal cells of *Lymnaea stagnalis* and the bag cells of *Aplysia californica* are the neuronal clusters controlling egg laying. Both cell clusters exhibit a discharge that initiates the behaviour (Ter Maat *et al.*, 1986; Dudek *et al.*, 1979). To establish the behavioural importance of these identified neurones they should be necessary and sufficient (Parsons *et al.*, 1983). Their sufficiency has been confirmed by electrically stimulating the neurones in intact

animals, which resulted in egg laying behaviour (Ter Maat *et al.*, 1989; Ter Maat & Ferguson, 1996). Their necessity has been tested by selective ablation, but only in *Aplysia brasiliana*. It was found that egg laying frequency severely decreased after lesioning the bag cells (Pinsker & Dudek, 1977), but it was not completely eliminated, showing that these neurones are not absolutely necessary although they play an important role.

During the discharge, the caudo-dorsal cells and the bag cells release several hormones into the blood, including the egg laying hormone (respectively: Geraerts *et al.*, 1985; Chiu *et al.*, 1979). When injected into the blood, the purified hormones evoke egg laying behaviour (respectively: Ter Maat *et al.*, 1989; Chiu *et al.*, 1979). It has also been shown that, besides the central control of egg laying, neuronal feedback from the reproductive organs is necessary for co-ordinating part of the behaviour. When the neuronal information from the eggs passing through the reproductive tract is removed by denervating the reproductive tract, parts of the egg laying behaviour are omitted (*Lymnaea stagnalis*: Ferguson *et al.*, 1993; *Aplysia fasciata*: Ter Maat & Ferguson, 1996).

The above shows that by using the *in vivo* techniques one is able to test the predictions that are made from *in vitro* experiments, and the electrical activity of a small population of neurones can be directly related to a natural behaviour. Bullock (1999) argued that the micro-stimulation technique has been particularly overlooked as a powerful method in neuroethological research. Additionally, micro-injections of the neuropeptides or neurotransmitters contained in the neurones - to mimic the natural release of these substances - can evoke the behaviour (Bullock, 1999). Combining these techniques with electrophysiological recordings during naturally occurring behaviour can provide conclusive evidence of the involvement of particular neurones in a behavioural event.

In Chapter 6, The *in vivo* recording and stimulation technique is used to test the involvement of the right mesocerebrum in mating behaviour, with special emphasis on dart shooting and penial eversion. Also, the proposed functions of APGWamide and FMRFamide are tested by introducing these neuropeptides into the blood. The results are discussed in an evolutionary context by comparing the data with those available from other molluscs.

Objectives of this thesis

In this thesis, I attempt to understand both the function of dart shooting and the means by which dart shooting is expressed by the central nervous system. Therefore, different research techniques are applied. In Chapter 2, I use behavioural observations and atomic absorption spectrophotometry to overturn the previous belief that the dart is a gift of calcium. In Chapter 3, I investigate the hypothesis that a biochemical substance in the mucus carried by the dart causes an effect on the recipient. I report that the mucus affects the physiology of the female reproductive system, which leads me to speculate that the dart serves to manipulate the partner to the shooter's advantage. However, the observed effects of the mucus can also be explained as mate choice by the recipient. I deal with this possibility in Chapter 4, in which I apply the evolutionary game theory to dart shooting. The developed game of darts for snails makes different predictions for the two alternative hypotheses. These predictions are subsequently tested, and the data are found to support the mate manipulation hypothesis. In Chapter 5, I briefly review the introduction of bioactive substances (in other species) that induce direct responses in the recipient's physiology, and I propose to use the term "allohormones" for such substances.

Having come to a better understanding of why darts are shot, I change the focus to neuroethology in Chapter 6, in which I try to learn how dart shooting and the other mating behaviours are controlled by the central nervous system. Using electrophysiological *in vivo* techniques I show that the mesocerebrum is the main control centre for mating behaviour, including dart shooting and penis eversion. A comparison with similar work done in other mollusc species suggests that this brain area has an evolutionarily conserved function in distantly related molluscs.

Chapter 7 is a general discussion of some of the issues brought up in the earlier chapters and a guide for future research on dart shooting and mating behaviour in snails.

[Figure on next page]

Figure 1. Dart shooting in the garden snail *Helix aspersa*. The photograph shows a pair of snails in the copulatory phase. A white love dart can be seen sticking out of the left side of one of the snails. This dart was shot by the snail's partner into the right side; it was stabbed so forcefully that it exited on the left side!

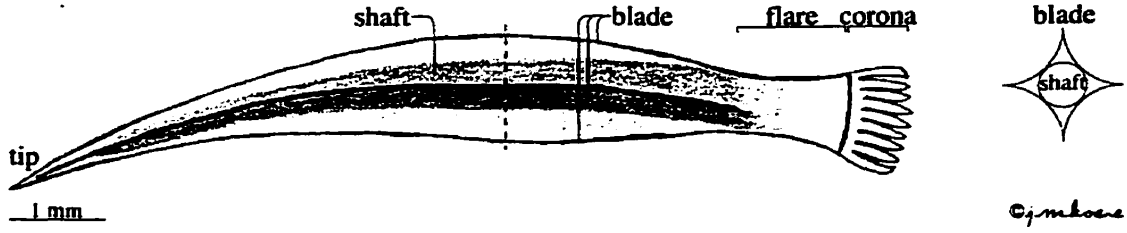

Figure 2. The love dart of the garden snail *Helix aspersa*. The dart is nine millimetres long and composed of calcium carbonate ($CaCO_3$) in the form of micro-crystalline aragonite. The shaft of the dart is hollow and filled with a gelatinous substance. The cross section shown on the right is indicated by the dashed line in the side view of the dart. The line between the flare and the corona indicates where the dart normally detaches when it is shot.

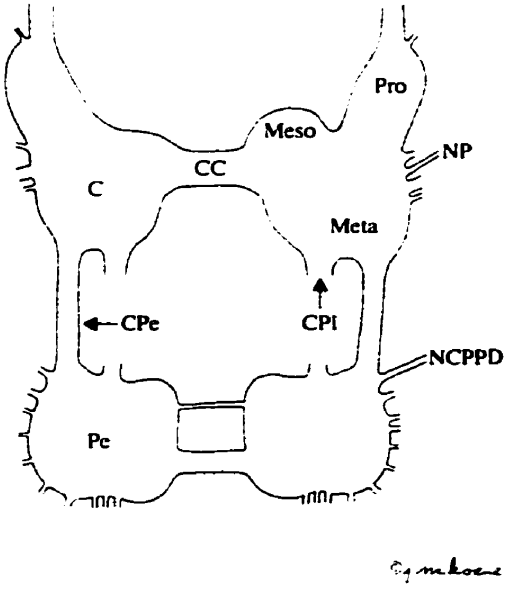

Figure 3. The central nervous system of the garden snail *Helix aspersa*. The drawing shows the cerebral ganglia (C) and pedal ganglia (Pe) that are connected by the cerebro-pedal connective (CPe). The cerebro-pleural connective (CPl) connects the cerebral ganglia to the other suboesophageal ganglia (not shown). Abbreviations: CC, cerebral commissure; Meso, mesocerebrum; Meta, metacerebrum; NCPPD, *nervus cutaneus pedalis primus dexter*; NP, *nervus penis*; Pro, procerebrum.

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Chapter 2

THE LOVE DART OF *HELIX ASPERSA* MÜLLER IS NOT A GIFT OF CALCIUM

In this chapter, I test the long-standing hypothesis that the love dart of the terrestrial snail serves as a nuptial gift of calcium. The dart of most species is mainly composed of a crystalline form of calcium carbonate. The nuptial gift hypothesis is based on the fact that for *Helix aspersa*, as for many other snail species, calcium is an essential element for both egg laying and growth. Eggs contain calcium in their shells and the embryo takes up this calcium during development to form the embryonic shell. Can the calcareous dart contribute to the large amount of calcium needed for egg production?

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THE LOVE DART OF *HELIX ASPERSA* MÜLLER IS NOT A GIFT OF CALCIUM

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ABSTRACT

The phenomenon of dart shooting in several species of land snails has still not been explained. We were interested in whether the dart can function as a nuptial gift of calcium, as previously proposed. Donating calcium would increase the fitness of the offspring and thereby result in a higher reproductive success for the donor. We confirmed in *Helix aspersa* that the developing embryo takes up calcium from the egg shell for the formation of its embryonic shell. However, other results from behavioural observations and calcium measurements in various reproductive structures do not support the calcium hypothesis. We found that the dart penetrated the skin in 91.7% of the shootings, but it was internalized by the recipient in only 6.3% of the shootings. The amount of calcium in one dart is roughly equal to that of one egg, and thus it would not contribute significantly to an average clutch of 59 eggs. The spermatophore contains virtually no calcium, and therefore it is unlikely that the dart signals a donation of calcium with the sperm. The dart is also unlikely to influence egg laying since dart shooting does not predict either the latency or the productivity of egg laying in the shooter or the recipient. We conclude that the love dart of *Helix aspersa* is not a gift of calcium. Instead, we suggest that it is a vehicle to introduce a substance into the partner to influence the fate of the donated sperm.

INTRODUCTION

Several species of land snails make use of a dart during mating. The so-called love dart is a hard structure, made of the calcium crystal aragonite (Tompa, 1984), which is pushed through the skin of the partner during courtship. The reason for this extraordinary behaviour has still not been determined, although many hypotheses have been proposed (reviewed by Kothbauer, 1988). In this study we are interested in whether the love dart can serve as a nuptial gift of calcium for egg production (Charnov, 1979; Leonard, 1992).

One of the species expressing dart shooting behaviour is the brown garden snail *Helix aspersa* (Müller). The simultaneous, reciprocal mating behaviour of this snail can be divided into three phases, namely introduction, dart shooting and copulation (Adamo & Chase, 1988). At the end of the introductory phase one of the snails in a courting pair shoots its dart, and the second snail usually shoots its own dart within 30 minutes (Adamo & Chase, 1988). The term "dart shooting", while convenient, is inaccurate because the dart does not actually fly through the air. The dart is pushed into the skin of the partner by a quick and forceful eversion of the muscular dart sac, in which the dart is produced and stored. The outside of the dart is covered with mucus from the digitiform glands that empty into the dart sac. Treatments of this behaviour in the literature generally assume that once the dart is shot it detaches from the dart sac and remains in the skin of the partner. It is further assumed that the shot dart is incorporated into the recipient (Charnov, 1979; Leonard, 1992). The dart is usually shot into the partner's right side, a few millimetres behind the partially everted genital atrium (Giusti & Lempri, 1980; Chung, 1987).

Once both snails have shot their darts they attempt to achieve a simultaneous intromission. The first dart shooter will already be attempting intromission even before the second dart is shot, but the partner will not allow intromission until it itself has shot a dart and is ready to intromit. After successful intromission the pair becomes motionless and maintains reciprocal intromission for 4 to 6 hours. This time is required to exchange the spermatophores which package the sperm (Adamo & Chase, 1988).

After mating, the snails lay eggs within a variable period of time. The egg clutches of *Helix aspersa* comprise of 50 to 100 eggs (Tompa, 1984), and they have a semi-calcified shell, which means that calcium crystals are embedded in a jelly-like inner layer of the shell (Tompa, 1984). It has been observed for

several other species of snails that the embryo takes up this calcium from the egg shell during development and uses it for the formation of the embryonic shell (*Anguispira alternata*: Tompa, 1975; *Stenotrema leai*: Tompa 1979; *Veronicella ameghina*: Tompa, 1980).
Crowell (1973) showed that calcium is one of the limiting factors for development, growth and reproduction in *Helix aspersa*. This fact, together with those mentioned above, suggests that a large amount of calcium needs to be provided to the eggs for survival. Thus, donating calcium to the mating partner, as a nuptial gift, could be a way to increase the fitness of the offspring. The concept of a nuptial gift is well known from studies of insect reproduction, e.g. the sodium donation by the male moth to increase the fitness of its offspring (Smedley & Eisner, 1996). The main question that we ask here is whether the dart is such a gift of calcium. This idea was first suggested by Charnov (1979) and has remained an attractive explanation for the dart shooting phenomenon (Leonard, 1992). An alternative hypothesis is also tested here, namely that the dart is a signal that the animal possesses a large supply of calcium and that it will transfer some calcium with the spermatophore. This idea, that the dart may function to entice the partner before the actual nuptial gift is donated, also comes from insect biology. The male moth of the species *Neopyrochroa flabellata* donates a small precopulatory amount of cantharidin (Spanish fly) and transfers more of it with the sperm (Eisner, Smedley, Young, Eisner, Roach & Meinwald, 1996a). The cantharidin is then transferred to the eggs and protects them from predators (Eisner *et al.*, 1996b). In this case the spermatophore contains the actual gift. The possibility that a similar scheme is operative in snails is supported by the fact that the spermatophore wall is impregnated with calcium salts in some species (Tompa, 1984).

METHODS

Specimens of the garden snail *Helix aspersa* (Müller) were obtained from Santa Barbara, California. Snails were only selected for study if they had a reflected shell lip, indicative of sexual maturity (Chung, 1987). They were aroused from aestivation and kept in individual Lucite boxes (7 × 8 × 8 cm). In most cases, they were fed lettuce, carrot roots and crushed oyster shells (as a source of calcium) *ad libitum*. Some snails (no-calcium group) were deprived of calcium for 8 months, beginning immediately after they shot a dart. The boxes were kept moist by adding water every day and they were cleaned every day or every two days. Whenever the boxes were cleaned, the snails were given a shower to keep them moist and active. The temperature was maintained at 23 ± 2°C, with a light-dark cycle of 14/10h.

After 7 to 10 days of isolation, the snails were put in group boxes (18 × 18 × 8 cm) for observation during several hours every day, at the start of the dark period. Each group box contained 16 snails. The courtship and dart shooting behaviours were observed every 10 minutes. Whenever two snails formed a pair and were at a high level of genital eversion (Adamo & Chase, 1988) they were carefully removed from the group box and put into a pair box (7 × 8 × 8 cm) for closer observation. The transfer to another box did not affect their mating behaviour. Whenever the typical dart shooting posture was observed the snails were watched until the dart was shot. With this protocol no dart shooting events went unobserved.

Darts that were lost during the mating trials were collected for calcium measurements. To obtain spermatophores for calcium measurements, copulating snails were separated just prior to the transfer of the spermatophore. The spermatophore is formed immediately after intromission but it is not transferred until 4.5 hours after formation (Adamo & Chase, 1988). When snails are separated before sperm transfer they expel the untransferred spermatophore through the genital pore within an hour (Lind, 1973), thus allowed for collection. In a few cases the spermatophores were surgically removed from the bursa tract diverticulum after transfer.

Eggs were obtained by placing snails in individual boxes (7 × 8 × 8 cm) that were lined with 3 cm of moist sand. The eggs that were collected for analysis were either left intact or the egg shell was separated from the embryo and the albumen fluid. Separation was achieved by carefully peeling off the egg shell with a pair of fine forceps.

To measure calcium, a Perkin Elmer 3100 atomic absorption spectrophotometer was used. The spectrophotometer was fitted with a hollow cathode lamp with an operating current of 12 mA, and it was set for the calcium specific wavelength of 422.7 nm. All glassware was pre-treated with 15% nitric acid (HNO_3) in distilled water to prevent calcium contamination. The biological tissues were dissolved in 5 ml of 70% HNO_3. After 2 hours of digestion, a 0.1 ml sample was taken and diluted in distilled water to a resulting volume of 10 ml. Each sample was measured three times. A range of standards was made from a 100 ppm calcium stock solution in the same HNO_3 matrix as the biological samples.

RESULTS

Dart shooting

To examine the reliability of dart shooting, mating snails were categorized by their dart shooting behaviour and divided into those that shot a dart and those that did not shoot a dart.

In Table 1 the results are reported as absolute numbers, as percentages of the total darts available and as percentages of the total darts shot. From the snails that shot a dart (60%), a further categorization was based on the dart's ultimate destination. Darts that did not hit the partner and were either lost on the ground or retracted into the dart sac were defined as missed; these were observed in 8.3% of the dart shootings. A majority of the darts, 85.4%, were categorized as temporary penetrations. These darts were shot into the skin of the partner and stayed there for several minutes to several hours, but were eventually lost. The remaining 6.3% of the darts were categorized as internalizations because they were retained by the partner for more than 24 hours.

Of the darts that penetrated the skin most (73.1%) were shot in the right side of the animal just posterior to the genital pore. Other darts were shot in the foot (23.1%) or the head (3.8%). These results are similar to those reported earlier by Giusti and Lempri (1980), Chung (1986), and Adamo and Chase (1988). The position where the dart was shot did not correlate with internalization of the dart. Also, the depth of a dart's initial penetration did not seem to predict its ultimate destination.

To test the effect of the availability of calcium on dart shooting, snails were deprived of calcium for 8 months, beginning immediately after they shot a dart. This caused a decrease in shell strength and in increase in deaths due to shell failure, indicative of an overall lack of calcium. In this group dart shooting occurred in 62.1% (18 of 33 individuals) of the mating snails, which is not significantly different (χ^2-test) from the result reported above for snails that had unlimited access to calcium.

Calcium contents

Table 2 shows the mean calcium content (± standard deviation) of the different tissues determined with atomic absorption spectrophotometry. The eggs for the calcium measurements were taken from 4 different clutches (10 eggs per clutch). The results revealed that, on average, one dart contains nearly the amount of calcium of one egg. A spermatophore only contains trace amounts of calcium. From the observation that the average egg clutch consisted of 59 ± 22 eggs, the total amount of calcium per clutch was calculated to be 24.25 mg. On average, the dart contains only 1.5% of this amount and the spermatophore contains even less (0.07%).

Measurements of the egg shell and the embryo were made from freshly laid eggs and from eggs that had been developing for 12 days and were close to hatching. The total amount of calcium per egg was calculated by adding up the measured amounts for the shell and the embryo. The calcium contents of the egg shell and the embryo are reported in Table 3 as mean percentages (± standard deviations) of the total calcium content. The eggs came from the same 4 clutches that were used in Table 2. The data in Table 3 show that a significant amount of the calcium of the egg shell is taken up by the developing embryo. When the eggs are freshly laid most of the calcium is located in the jelly-like inner layer of the egg shell in the form of calcium crystals. During development of the embryo there is a gradual uptake of calcium (data not shown). By the time of hatching, most of the calcium has been taken up by the embryo for use in the production of the embryonic shell.

Table 1. Reliability of dart shooting in *Helix aspersa*.

Snails	Not shot	Shot	Missed	Temporary penetration	Internalization
80	32	48	4	41	3
100%	40%	60%	5%	51.2%	3.8%
		100%	8.3%	85.4%	6.3%

Table 2. Calcium contents of various reproductive structures.

Dart (N=32)	Spermatophore (N=22)	Egg (N=40)	Clutch (59 eggs)
0.369 ± 0.131	0.018 ± 0.041	0.411 ± 0.139	24.25 (calculated)

Means ± standard deviations, in mg.

Table 3. Distribution of calcium in the egg during development.

Egg part	Fresh eggs (n=32)	Hatching eggs (n=34)	T-test
shell	80.85% ± 15.31%	32.58% ± 30.48%	P<0.001
embryo	19.15% ± 15.31%	67.42% ± 30.48%	P<0.001

Means ± standard deviations expressed as percentages of the total calcium content of the egg.

Table 4. Egg laying in relation to dart shooting and dart reception.

	Shot a dart		Did not shoot a dart		Total	No calcium	Control
	Received (N=18)	Not received (N=12)	Received (N=3)	Not received (N=10)	(N=43)	(N=11)	(N=12)
eggs	66±22	57±24	42±3	55±19	59±22	66±23	59±28
latency	22±21	28±25	11±7	16±30	22±24	6±5	11±8

Eggs: mean number of eggs per clutch ± standard deviation; latency: mean number of days from mating to egg laying ± standard deviation; total: grand mean of the experimental groups; no calcium: mated snails deprived of calcium; control: unmated snails.

Egg laying

The mean latency from mating to egg laying for all the experimental groups was 22 ± 24 days, with an average clutch size of 59 ± 22 eggs (N=43). Table 4 shows the mean number of eggs and the mean latency to egg laying for snails grouped according to whether they shot and/or received a dart. There was no significant difference (two-way ANOVA) between any two groups for either the number of eggs laid or the latency to egg laying. Also, comparison of the grand mean of all experimental groups with the controls showed no significant difference (t-test). In the no-calcium group 11 snails laid eggs, with an average clutch size of 66 ± 23 eggs and a latency of 6 ± 5 days (Table 4). There was no significant difference (t-test) for either the number of eggs laid or the latency to egg laying compared to the unmated controls. Also, comparison to mated controls (not shown in Table 4), which laid 63 ± 26 eggs with a latency of 9 ± 8 days, revealed no significant difference.

DISCUSSION

Calcium is one of the limiting factors for growth and reproduction in snails (Crowell, 1973). To test whether calcium could be donated during mating we made behavioural observations and measured calcium levels in different reproductive structures. We confirmed that the developing embryo of *Helix aspersa* takes up calcium from the shell, as had been reported for other snail species (Tompa, 1975, 1979, 1980). However, additional results do not support the calcium hypothesis.

Contrary to general assumptions in the literature, our observations of dart shootings show that most of the darts are not internalized by the recipient. Chung (1987) and Adamo and Chase (1988) also found that many dart shootings do not result in internalization. From the published data of these authors, the percentage of darts that were, and were not, internalized was calculated for comparison to our own data. Table 5 shows these comparative data, and it is evident that all three investigations reveal the same trend. The totals show that, although 82.6% of the darts pierce the skin, only 22.1% are internalized. Some of the differences in percentages between the previous studies and the present one may be due to different definitions of missed and internalized darts. For example, in the present study some darts were found to be expelled a day after mating; these may have been considered by previous authors as internalized because of shorter observation periods. Nevertheless, the inference that can be drawn from these data is that the dart is unlikely to be a gift of calcium since it is seldom internalized.

The calcium measurements are also inconsistent with the love dart being a gift of calcium because the results indicate that the dart does not contain enough calcium to contribute significantly to an egg clutch. On average, one dart

Table 5. Fate of the love dart.

	Shot (N)	Skin penetration	Internalization
Chung (1987)	42	66.6%	14.3%
Adamo & Chase (1988)	48	89.6%	45.8%
This paper	48	91.7%	6.3%
Total	138	82.7%	22.1%

Skin penetration: temporary penetrations plus internalizations (excludes misses).

(0.369 mg calcium) contains roughly the same amount of calcium as one egg (0.411 mg calcium). For an average egg clutch this would only be a contribution of about 1.5%. Also, the spermatophore contains virtually no calcium, indicating that the dart is unlikely to be a precopulatory gift with the spermatophore as the actual gift of calcium. In addition to these empirical arguments, it should be noted that a gift of calcium delivered via either the dart or the spermatophore would usually be reciprocal. Thus, a snail would receive and donate an equal amount of calcium. Neither of the mating partners would benefit from such a reciprocal donation.

It seems unlikely that the dart is a signal from the shooter indicating that it will soon lay eggs or that it has enough calcium for egg laying. If this were the case, a short latency from mating to egg laying might be expected. Instead, the latency to egg laying was long and variable. Nor did we find any difference in clutch size for snails that shot a dart compared to those that did not shoot. Lastly, the dart did not affect either the latency to oviposition or the number of eggs laid by the recipient, indicating that receipt of a dart does not trigger or influence egg laying. However, caution should be taken in interpreting these results because the sample sizes are small.

Any transmission of information about the size of a dart shooter's calcium store is irrelevant when calcium is freely available in the environment. Since egg production and oviposition only start once a successful nest has been excavated, which may require several attempts over several days (Tompa, 1984), the period between mating and egg laying allows ample time for the ingestion of calcium. This indicates that a snail does not need to ingest and store all the calcium necessary for egg production before mating. Therefore, there is no use for a dart to inform a potential mate of the shooter's calcium content.

In these experiments most of the factors that are thought to influence calcium uptake and egg laying (e.g. the availability of calcium, humidity, temperature and nutrition) were kept optimal (Daguzan, 1981). Conceivably, in some natural environments where calcium is not plentiful, and thus where post-copulatory calcium ingestion would be limited, the dart might convey useful information about the shooter's readiness to produce viable eggs. To test this possibility, the calcium deprivation experiment was performed. Since the results showed that the availability of calcium affected neither dart shooting nor egg laying, it seems unlikely that the dart functions differently in natural environments and laboratory experiments.

Why then would a snail produce and shoot a calcium dart if it is not used for the production of eggs or as a signal of fitness? The reason to produce such an expensive signal might be found in the conflict of interest between the male and female functions (Charnov, 1982). We favour the pheromone hypothesis that was suggested earlier by several authors (Dorello, 1925; Börnchen, 1967; Chung, 1986; Adamo & Chase, 1990, 1996). Accordingly, the dart might be used as a vehicle to introduce a substance into the partner that increases the chance that the shooter's sperm will be used to fertilize the recipient's eggs. This would explain why both snails shoot a dart, since both snails will benefit from influencing the fate of their sperm. Such a function for the dart would only require penetration of the skin, instead of full internalization, in order to transfer the substance to the partner. The economical construction (double-H beam construction; Hunt, 1979), which makes use of minimal material for maximum strength, is consistent with this idea.

Snails can store received sperm for long periods of time (Tompa, 1984), which increases sperm competition. A snail would have an advantage if it could get more of its donated sperm to escape from the spermatophore and reach the female system. Lind (1973) surmised that only 0.1% of the donated sperm reaches the female tract in *Helix pomatia*. We propose

that a substance in the mucus of the digitiform glands is transferred to influence the fate of the donated sperm. Lind (1973) reported that the mucus of the dart caused a dilation of the bursa copulatrix in *H. pomatia* where the spermatophore is received. In *Helix aspersa* the spermatophore is received in the bursa tract diverticulum, which contracts to pull in the spermatophore. If a pheromone in the mucus were to act, either directly or indirectly, to slow down the contractions of the bursa tract diverticulum, more sperm could escape through the tail canal of the spermatophore into the spermoviduct before the remainder was degraded in the bursa copulatrix. Another site where a factor in the mucus could have an effect is in the spermoviduct, where contractions occur to transport the received sperm to the spermatheca, the site where foreign sperm is stored. These ideas can be tested by experiments *in vitro*.

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Chapter 3

CHANGES IN THE REPRODUCTIVE SYSTEM OF THE SNAIL *HELIX ASPERSA* CAUSED BY THE MUCUS COVERING THE LOVE DART

After having demonstrated that the dart does not serve as a gift of calcium, I here test the hypothesis that the mucus that covers the dart causes an effect on the recipient's reproductive system. This hypothesis follows from research in which the effect of the dart on the recipient's behaviour was tested. As reported previously, the behavioural effect of the mucus is small, so it was proposed that the mucus might have an effect on the recipient's reproductive endocrinology rather than on its behaviour. To test this idea, instead of injecting the mucus into intact animals (as done in the previous studies), I chose an *in vitro* preparation that allowed me to directly observe and measure the effects of the mucus on the reproductive organs.

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CHANGES IN THE REPRODUCTIVE SYSTEM OF THE SNAIL *HELIX ASPERSA* CAUSED BY MUCUS FROM THE LOVE DART

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Summary

The function of the love dart in certain species of terrestrial snails is unknown. In *Helix aspersa*, the dart is a sharp calcareous structure that is used to pierce the partner's skin during courtship. When expelled, the dart is covered with a thick mucus. The hypothesis tested here is that the mucus contains a biologically active substance. Extracts of the digitiform glands that produce this mucus were applied to parts of the reproductive system *in vitro*. The extracts triggered an initial reconfiguration of the copulatory canal that caused the bursa tract diverticulum to become more accessible to the spermatophore. The reconfiguration of the copulatory canal also closed off the tract leading to the bursa copulatrix, a sperm-digesting organ. A few minutes after the initial contraction, the peristaltic contractions in the diverticulum became significantly more frequent. This latter effect continued for at least 1 h, provided that the mucus extract remained in the saline bath. The minimum effective dosage was less than the 2.2 mg of mucus transferred with the dart.

Sperm competition is expected in *Helix aspersa* since multiple matings occur before eggs are laid. By influencing the female organs involved in the processing of foreign sperm, the dart shooter may increase the chance that his sperm will fertilise eggs.

Key words: love dart, mollusc, snail, *Helix aspersa*, reproduction, sperm competition, mucus.

Introduction

Nature is replete with examples of bizarre mating behaviours. Among these is the shooting of a love dart in several species of land snails. The function of dart shooting has bewildered investigators since at least the time of Swammerdamm (1637–1680). Numerous hypotheses have been proposed (reviewed by Kothbauer, 1988), but none is yet proven. In the garden snail *Helix aspersa* dart shooting is expressed at the end of courtship behaviour (Adamo and Chase, 1988). The dart sac, which produces and stores the dart, is forcefully everted from the genital pore, causing the dart to be expelled. Typically, but not invariably, the shot dart perforates the skin of the mating partner. We use the term 'shot' loosely. Since the courting partners are in close contact, the dart does not fly freely.

One type of explanation for the dart derives from the fact that the dart is made of calcium, formed as the crystal aragonite (Tompa, 1980). Since calcium is important for the development of snails (Crowell, 1973; Tompa, 1980), it has been thought that the dart might be a nuptial gift of calcium for the production of eggs (Charnov, 1979). We recently refuted this hypothesis by demonstrating that an insignificant amount of calcium is transferred with the dart relative to the amount present in the eggs and, moreover, that the dart is seldom internalised by the recipient. We also found no experimental support for the related ideas that the dart might induce egg-laying in the recipient or that it might signal the readiness of the shooter to lay eggs (Koene and Chase, 1998). It remains possible that the dart serves some other signalling function (Leonard, 1992), but in our view this is unlikely (Adamo and Chase, 1996).

Another type of explanation assumes that the dart directly influences either the behaviour or the reproductive physiology of the mating partner. This idea has been suggested in several different forms. Many authors (Dorello, 1925; Börnchen, 1967; Chung, 1986; Adamo and Chase, 1990) have sought to detect an effect of the dart on sexual arousal, thus echoing the legends of Eros/Cupid from classical mythology (Kothbauer, 1988). Observations indicate, however, that the receipt of a dart has only a small behavioural effect on sexual arousal, as measured by the degree of genital eversion, and it only slightly shortens the duration of courtship (Chung, 1986; Adamo and Chase, 1990). The effect seems to be too small to account for such a costly behaviour.

It is important to note that the dart could stimulate the recipient either mechanically or chemically. As the dart is expelled from its storage sac, it is covered by a mucus that derives from a pair of digitiform glands. Earlier experiments have demonstrated the feasibility of the dart acting as a hypodermic device to deliver the mucus to the interior of the recipient. In fact, the arousing effects noted above were shown

to be equal whether the dart was shot normally or the mucus of the dart was injected by a method that minimised mechanical stimulation (Chung, 1986; Adamo and Chase, 1990). Furthermore, in the latter case, the stimulatory effect was lost when the mucus extract was treated with pronase (Chung, 1986).

Here, we test whether an effect can be observed in the reproductive system of *Helix aspersa* by applying extracts of the digitiform glands *in vitro*. Fig. 1 shows a schematic overview of the reproductive system. Although *Helix aspersa* is a simultaneous reciprocal hermaphrodite, we will occasionally refer to gender-specific roles. As can be seen from Fig. 1, all the organs have an opening into the genital atrium, which connects to the outside *via* the genital pore. The genital atrium is gradually everted during courtship and it becomes visible as a white bulge on the right side of the animal (Adamo and Chase, 1988). Following dart shooting, the penis is everted and each snail attempts to intromit its partner. Simultaneous intromission, required for successful copulation, is achieved when the penises of both snails are inserted into the copulatory canals of the partners (Tompa, 1984). At this point, the spermatophore is formed in the epiphallus and the flagellum, and it is filled with sperm from the hermaphroditic duct *via* the vas deferens. When the spermatophore is complete, it is transferred into the bursa tract diverticulum of the partner, after which the snails separate. The sperm escape from the spermatophore through its tail canal to enter the female tract. They travel up the spermoviduct to reach the spermathecal sacs, where they are stored prior to being used for fertilisation (Lind, 1973). The spermatophore and the remaining sperm are transported through the bursa tract into the bursa copulatrix, where they are digested (Lind, 1973).

Snails mate with several partners during a season before they lay eggs (Tompa, 1984). From each mating, it is estimated that only approximately 0.1 % of the donated sperm actually reach the spermathecal sac; the rest are digested in the bursa copulatrix (*H. pomatia;* Lind, 1973). For these reasons, it would be advantageous for a sperm donor to increase the survival and utilisation of his sperm relative to that of his competitors. The experiments described here suggest a possible role for the dart in sperm competition.

Materials and methods

Specimens of *Helix aspersa* Müller were obtained from Santa Barbara, California. The snails were kept at 20–25 °C with a light:dark cycle of 16 h:8 h. They were fed lettuce, carrots and crushed oyster shells. To increase sexual proclivity, the snails were isolated for at least 10 days prior to use. Thereafter, groups of 10–20 snails were occasionally placed in a small box (18 cm×18 cm×8 cm) for observation. Pairs of snails were selected for experimentation when they exhibited courtship behaviour (Koene and Chase, 1998).

To confirm that mucus is transferred with the dart, the masses of darts were measured before and after shooting. Shot darts were collected and weighed immediately after shooting (in cases where they missed the intended recipient) or later, after they had been sloughed off by the recipient. In the latter cases, we used only darts that had penetrated the skin for 25 % or less of their length, to avoid losing mucus inside the recipient. The masses of darts before shooting were determined by dissecting darts from the dart sacs of sexually active snails. After the wet mass had been measured, the darts were lyophilised to determine their dry mass.

The digitiform glands were dissected out of sexually active snails. Extracts of the glands were made in a hand-held homogeniser with 0.5 ml of saline solution. Extracts of the columellar retractor muscle were used as a control for muscle tissue, and extracts of the pedal gland were used as a control for non-specific effects of mucus. The quantity of control tissue homogenised was comparable to that of the digitiform glands. To test effects from soluble components of recently shot darts, these were collected immediately after shooting and incubated in saline for at least 1 h at room temperature (20–25 °C).

In initial experiments, the entire reproductive system (Fig. 1) was dissected out and tested. Later, the preparations were reduced to include only the genital atrium, the copulatory canal and the bursa complex. The bursa complex comprises the

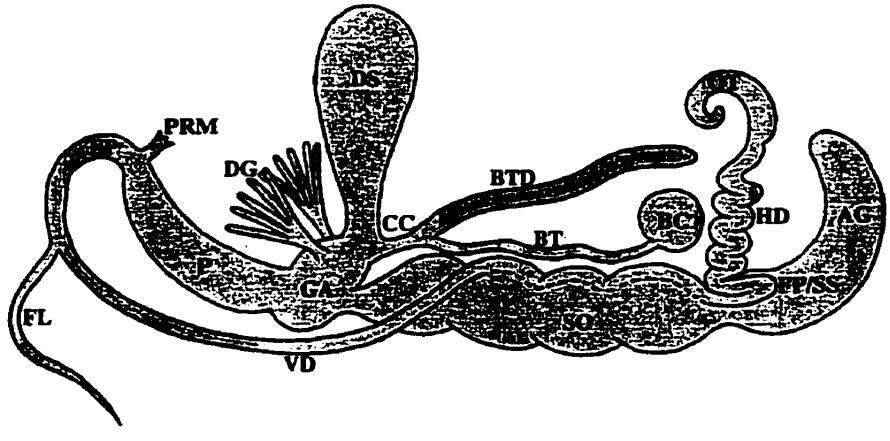

Fig. 1. Schematic drawing of the reproductive system in *Helix aspersa*. AG, albumen gland; BC, bursa copulatrix; BT, bursa tract; BTD, bursa tract diverticulum; CC, copulatory canal; DG, digitiform glands; DS, dart sac; EP, epiphallus; FL, flagellum; FP/SS, fertilisation pouch/spermathecal sacs; GA, genital atrium; HD, hermaphroditic duct; OT, ovotestis; P, penis; PRM, penis retractor muscle; SO, spermoviduct; VD, vas deferens.

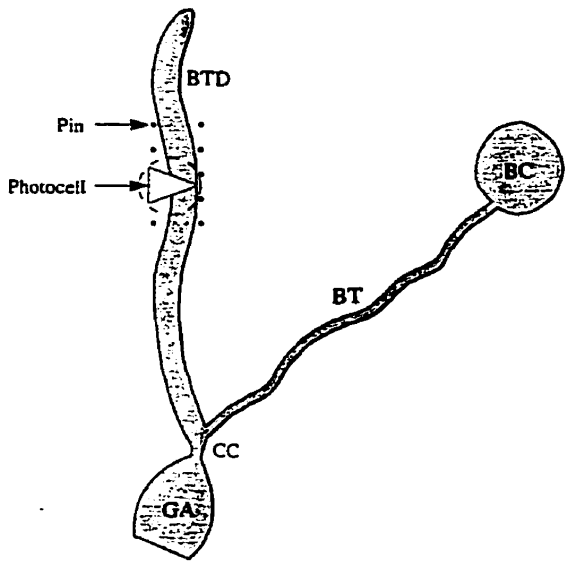

Fig. 2. Arrangement for recording contractions in the reproductive organs. The preparation was placed in a small dish and covered with 2 ml of saline. A triangular photocell was placed over the organ of interest, with a small light source coming from underneath, as indicated by the broken circle. Several pins were placed around the organ to prevent it from moving out of the field of measurement. For abbreviations, see Fig. 1.

bursa tract diverticulum and the bursa tract leading to the bursa copulatrix. These tissues were placed in a dish containing 2 ml of saline solution. Fig. 2 shows the experimental arrangement for measuring the responses of the copulatory canal and the diverticulum (shown here only for the diverticulum). A photocell was placed over the organ of interest, with a light source coming from underneath. When the organ contracted, there was a change in light intensity measured by the photocell and registered on a chart recorder. The photocell was preferred over a force transducer because the contracting diverticulum tended to pull in multiple, unpredictable directions. Movements of the organ were restricted by several pins inserted into the Sylgard base of the dish to prevent displacement of the organ out of the range of the measurement system.

For each trial, the maximum and minimum levels of the output of the photocell were determined, as shown in Fig. 3. Contractions of the organ were scored if their amplitude exceeded 25 % of the difference between the minimum and maximum levels. Peaks had to be separated by at least 30 s, otherwise they were counted as single occurrences. If the trace remained above the criterion level for more than 3 min, the event was not scored as a contraction and a new criterion level was established. Fig. 3 shows an example of these procedures; criterion-level contractions are labelled with an asterisk.

Basal (control) activity was recorded for 10 min before a test solution was added to the bath (Fig. 3). After the test solution had been added, the extract was allowed to take effect for 5 min, then activity was recorded for another 10 min (response period). The number of contractions was counted during the 10 min control period and during the 10 min response period. To calculate the overall response, the number of contractions in the control period was subtracted from the number in the response period. For the dose–response curve, each of 10 preparations was tested using six different test solutions. At the end of each trial, the contents of the saline bath were exchanged twice with fresh saline to remove the test solution. There was a 5 min rest period between each trial. Some preparations were tested with digitiform gland extract as well as with control substances. When single preparations were tested with either multiple doses or with multiple types of material, the order of testing was varied systematically. To avoid pseudoreplication, each dose or type of extract was used only once in any single preparation, and no test result was included in more than one figure or more than one statistical analysis. All records were coded before analysis.

Several other preparations were recorded for a longer time. In these cases, the control level activity was recorded for 10 min before the extract was added, followed by 60 min of response time. The recording was then interrupted for 1 min to wash out the extract, and the recording was continued for another 20 min. The number of contractions was counted in 10 min bins.

To test whether there is an effect of dart shooting on the success of spermatophore transfer, observations were made of dart shooting events during courtship. Particular attention was paid to whether the dart penetrated the skin. At 16–20 h after the beginning of copulation (10–16 h after the end of

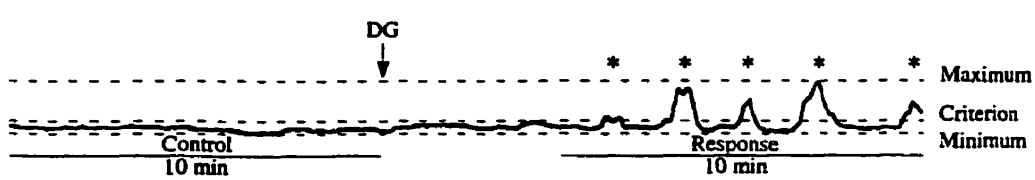

Fig. 3. Procedure for scoring contractions of the bursa tract diverticulum. The criterion level was set at 25 % of the difference between the minimum and maximum levels. Additional criteria were that individual contractions of the appropriate amplitude had to be separated from one another by at least 30 s and should not exceed 3 min in duration. The asterisks indicate contractions that were counted in this record. For each trial, the response to the application of a test substance (here, digitiform gland extract, DG) was measured as the number of contractions counted in the response period minus the number counted in the control period.

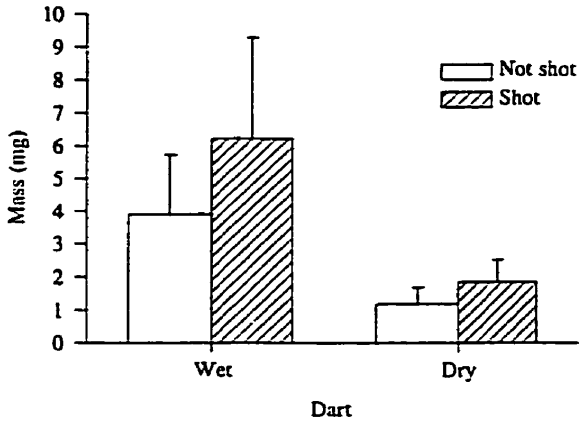

Fig. 4. Masses of the dart before and after shooting. The darts collected before shooting (not-shot, N=14) were obtained by operating on sexually active animals. The shot darts (N=15) were collected from snails of the same group after they had been expelled. The mean masses, wet and dry, are shown with their standard deviations. In both cases, shot darts were significantly heavier than not-shot darts (P<0.05; two-way ANOVA with Bonferroni correction).

copulation), the snails were examined to determine the extent to which the spermatophore had been transferred.

Results

Shot darts weigh significantly more than not-shot darts (Fig. 4), in both wet and dry conditions (P<0.05; two-way analysis of variance, ANOVA, with Bonferonni correction). The difference between the mean wet masses is 2.2 mg.

The tissue extracts were first tested on the entire reproductive system. We observed the induction of contractions in several organs including the penis, the spermoviduct and the genital atrium. However, with the exception of those in the copulatory canal and the bursa tract diverticulum, all the induced contractions were of short duration (<2 min). We therefore reduced the preparation to focus on effects in the copulatory canal and the bursa tract diverticulum. When monitored using a photocell, the copulatory canal shows an initial large movement in response to the application of digitiform gland extract followed by a prolonged change of position (Fig. 5A). From visual observations, we could see that the induced contractions closed off the entrance to the bursa tract while opening up the entrance to the diverticulum (Fig. 5B). This was confirmed by probing the two organs just before, and 5–10 min after, addition of the glandular extract. The probing was performed with a small piece of Plasticine shaped like the widest part, i.e. the body, of the spermatophore. Prior to the addition of the extract, the bursa tract was easily accessed from the copulatory canal while the diverticulum was obstructed. The situation was reversed after the addition of the extract, with the diverticulum now being more accessible than the bursa tract. The effect on the copulatory canal was confirmed by photocell recordings in 20 of 22 preparations.

In contrast to the early effect on the copulatory canal, the digitiform gland extract influenced the bursa tract diverticulum only after a latency of several minutes (Fig. 5A). The nature of the effect in the diverticulum was also different because, in this organ, the extract either initiated peristaltic contractions or increased their frequency. The potency of the extract was established using dose–response tests (Fig. 6). Doses of 0.03–0.05 ml caused increases in peristaltic rate that were significantly greater than those caused by the saline control (P<0.05; two-tailed Mann–Whitney U-test with Bonferroni correction), whereas lower doses were not significantly effective.

The duration of the effect was tested by recording from several preparations for a longer period. In these experiments, in which the digitiform gland extract remained in the saline bath for 60 min before being washed out, we found that the rate of peristalsis remained significantly elevated for at least 30 min (Fig. 7).

To test the specificity of the digitiform gland extract in producing changes in contraction rates, additional extracts were prepared from several tissues (Table 1). Responses to these extracts were determined in the same manner as previously described. Extracts of the columellar retractor muscle were consistently ineffective in increasing contraction rates, while extracts of a shot dart (which contained digitiform gland mucus) were always effective. These results suggest that the active material in the digitiform gland extract is mucus, not muscle or connective tissue. We tested this idea by using extracts of the pedal gland, which secretes mucus onto the sole of the foot as an aid to locomotion. In 68 % of the preparations tested, pedal gland extracts were effective in producing an increase in the contraction rate of the diverticulum. By comparison, extracts of the digitiform glands were effective in 84 % of the preparations. The difference in reliability of the two extracts is significant (P<0.005; χ^2-test). To compare the rate increases caused by pedal gland extracts and by digitiform gland extracts, the two substances were tested alternately in the same preparations (N=8). The mean increases (± S.D.) in the

Table 1. *Specificity of the substance inducing contractile changes in the bursa tract diverticulum*

		Percentage of preparations	
Extract	N	Rate increase	No rate increase
Digitiform glands	62	83.9	16.1
Pedal gland	22	68.2	31.8
Columellar retractor muscle	5	0	100
Shot dart (with mucus)	6	100	0

Rate changes were measured as described in Fig. 3. All rate increases, regardless of magnitude, are indicated here. In none of the preparations was a rate decrease observed.

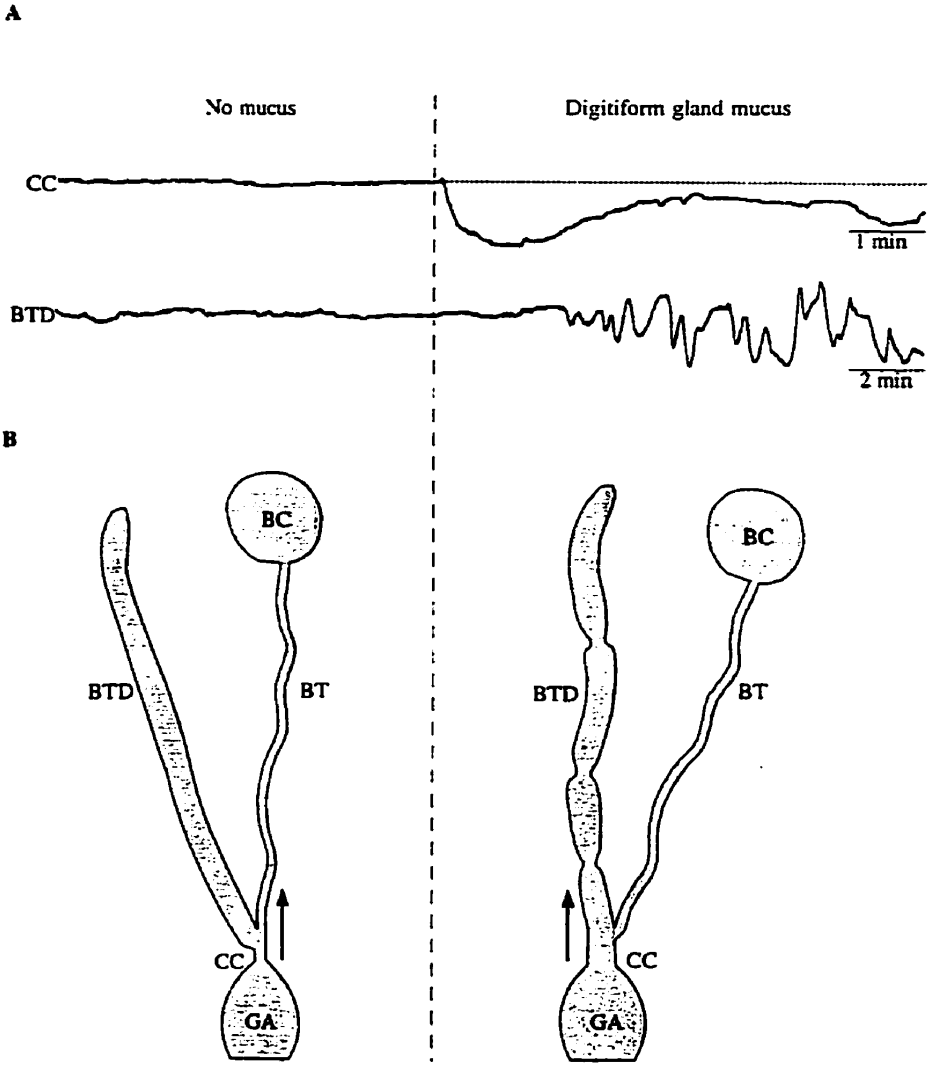

Fig. 5. Summary of the effects of digitiform gland mucus. (A) Sample traces for the copulatory canal (CC) and the bursa tract diverticulum (BTD). The dashed vertical line indicates the time of application of the extract. The dotted horizontal line provides a reference for the new configuration of the copulatory canal after application of the extract. Note the different time scales for the two traces. (B) Schematic representation of the observed effects. When no mucus is present, the copulatory canal connects to the bursa tract as indicated by the arrow. When the digitiform extract is added, the copulatory canal reconfigures to close off the bursa tract and make the bursa tract diverticulum more accessible (indicated by displacement of the arrow). Also, as indicated by crimping, peristaltic movements begin in the diverticulum or their rate increases. For other abbreviations, see legend to Fig. 1.

number of contractions per 10 min period were 2.88±1.96 with pedal gland extract and 3.31±2.46 with digitiform gland extract. Since these means are not significantly different (P=0.277; paired t-test), we conclude that the two extracts were equally potent, but unequally reliable.

We observed mated pairs to determine whether the spermatophores had been successfully transferred. In the majority of cases no part of the spermatophore was visible 16–20 h after the start of copulation, and it was assumed to have been incorporated. However, in some cases the tail of the spermatophore was still visible protruding from the genital pore. This was observed in only 2.5 % of the snails that received a dart (N=118), but in 13 % of the matings in which the dart did not penetrate the skin (N=92). Thus, complete transfer of the spermatophore is associated with successful dart shooting significantly more often than it is with unsuccessful dart shooting (P<0.001; χ^2-test).

Discussion

The mucus of the digitiform glands causes two important changes in the female portions of the reproductive system, as illustrated in Fig. 5. First, there is an early reconfiguration of the copulatory canal that begins immediately after the digitiform gland extract is added. Second, there is a delayed induction or potentiation of peristalsis in the bursa tract diverticulum that begins after a latency of several minutes.

The contractions in the copulatory canal seem to close off the entrance to the bursa tract and make the bursa tract diverticulum more accessible. Since the spermatophore is transferred into the diverticulum, transfer could be facilitated when the entrance to the diverticulum is widened. More importantly, the bursa tract leads to the bursa copulatrix, where excess sperm is digested. When the route to the bursa copulatrix is closed off, digestion of sperm would be delayed

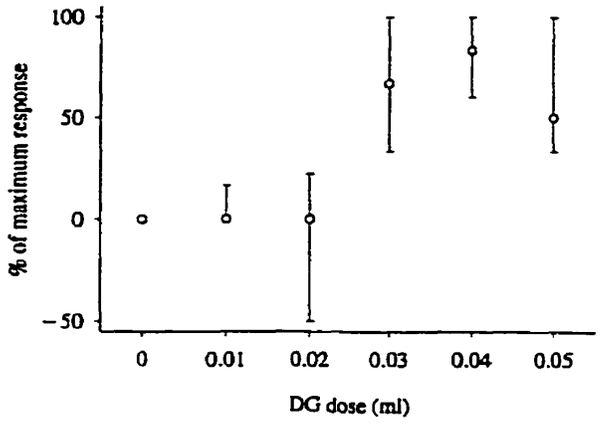

Fig. 6. The dose–response curve for the effect caused by the digitiform gland (DG) extract on the bursa tract diverticulum. A measure of response was calculated for each dose by taking the difference between the number of contractions observed in the control period and the response period (see Fig. 3). Ten preparations were tested with five doses. For each preparation, the response at a given dose was calculated as a percentage of the maximum response for that preparation regardless of dose. The graph shows the medians of these normalised responses, with error bars indicating the twenty-fifth and seventy-fifth percentiles. Maximum response values range from 2 to 9, with a mean of 5.0. Doses of 0.03–0.05 ml caused responses significantly greater than the saline control (0 ml), but doses of 0.01 and 0.02 ml had no significant effect ($P<0.05$; two-tailed Mann–Whitney U-test with Bonferroni correction).

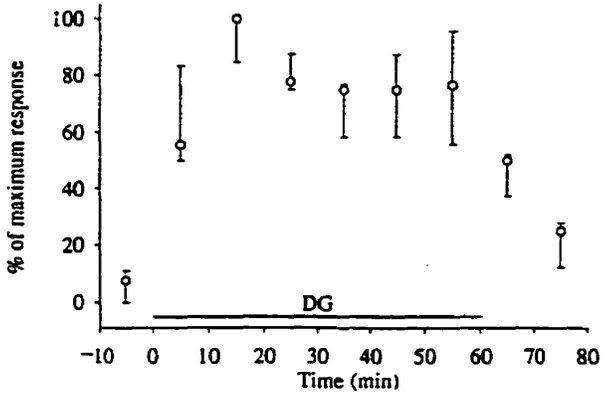

Fig. 7. Persistent influence of digitiform gland (DG) extract on contractile activity in the bursa tract diverticulum. Digitiform gland extract (0.05 ml) remained in the saline bath for 60 min as indicated by the horizontal bar. Response values were calculated as in Fig. 6. The bin width is 10 min, with medians and error bars (twenty-fifth and seventy-fifth percentiles) from five preparations plotted in the middle of the bins. Maximum response values range from 4 to 8, with a mean of 5.8. The responses recorded in the period 10–30 min following application of the extract are significantly greater than those recorded in the period from −10 to 0 min, i.e. before application ($P<0.05$; two-tailed Mann–Whitney U-test with Bonferroni correction).

and more sperm would have the opportunity to reach the spermathecal sacs.

The increased peristaltic contractions of the diverticulum should produce consequences that are synergistic with those noted above. Because the bursa tract is initially closed off by the reconfiguration of the copulatory canal, the faster transfer brought about by increased peristalsis could allow more time for the sperm to escape from the spermatophore and reach the female tract before the copulatory canal resumes its normal configuration.

The effect of digitiform gland extract on peristalsis in the diverticulum was persistent. We found that there was a statistically significant increase in contraction rate for 30 min following application of the extract, and a substantial increase, albeit statistically insignificant, for an additional 30 min, provided that the extract remained in the bath (Fig. 7). Normally, when the mucus is introduced into the blood by the dart, it will remain there until broken down or sequestered. Our results suggest that the mucus retains its biological activity throughout the period of circulation in the blood. A long-lasting effect would be needed to influence spermatophore transfer because complete transfer requires 4.5–6 h (Adamo and Chase, 1988). The idea that dart shooting influences transfer of the spermatophore is supported by our observations of animals after copulation. Most of the animals that were found to have a spermatophore tail still external to the genital pore many hours after the end of copulation were animals that had not been penetrated by a dart. Successful dart shooting, here defined as penetration, reduced by more than fivefold the percentage of matings wherein transfer of the spermatophore was incomplete.

There is reason to think that the concentrations of extract that were effective in our experiments are biologically significant. The blood volume of *Helix aspersa* is estimated to be 2.0–2.5 ml (Martin *et al.* 1958; Chung, 1985). This is equivalent to the volume of the saline bath used in the experiments (2 ml). Since the pair of donor glands from a single snail was diluted in 0.5 ml of saline, the highest dose (0.05 ml) represents 10 % of the digitiform glands. Given that the mean mass (± S.D.) of the glands is 22.7±8.5 mg (N=39), the highest dose contained 2.27 mg of gland material, not all of which consisted of mucus. From the data shown in Fig. 4, we conclude that the dart carries approximately 2.2 mg of mucus (see also Chung, 1986). Thus, the amount of mucus contained in our highest dose is approximately equal to, or even less than, the amount transferred by the dart. Consistent with this conclusion, extracts made from shot darts (with mucus) caused effects that were statistically indistinguishable from those caused by extracts of the digitiform glands (Table 1).

It is noteworthy that a difference in mass between shot darts and not-shot darts is apparent regardless of whether the measurements are made before or after dehydration (Fig. 4). This result is surprising because in both cases the difference

must be attributed to the mucus. Other molluscan mucuses are reported to contain at least 90% water (Denny, 1983). Our results suggest that mucus from the digitiform glands might be exceptional in containing less than the usual amount of water, but presumably more of a biochemical product that has the allohormonal function implicit in our observations.

The active substance may be a general constituent of mucus since extracts of the pedal gland also increase the rate of contraction of the diverticulum (Table 1). Alternatively, it is possible that the two extracts cause the same effect through different mechanisms. While both extracts cause rate increases, they may differ in variables that were not measured, such as the amplitude or shape of contractions. Regardless, it is clear that a priority for future research is the identification of the active substance(s) in the mucus of the digitiform glands and the pedal gland. Chung (1986) reported data suggesting that the active substance in the mucus is a polypeptide with a molecular mass of approximately 5000.

We conclude that the dart functions as a vehicle to transfer mucus from the digitiform glands into the mating partner. Further experiments will have to be performed to test our hypothesis that the net effect is to increase the survival of sperm transferred by the shooter to the female tract of the recipient. Our hypothesis implies that the dart shooter manipulates his partner to increase his own reproductive success. By influencing the female organs involved in the receipt and transport of foreign sperm, the shooter can increase the chances that his sperm will fertilise the eggs of the recipient. Since multiple matings occur before eggs are laid, sperm from different males are expected to compete for fertilisation of the eggs. According to this idea, the dart evolved as a result of sperm competition.

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Chapter 4

THE LOVE DART OF HERMAPHRODITIC SNAILS: MATE CHOICE OR MATE MANIPULATION?

Much research has been done on competitive aspects of sexual selection in animals with separated sexes. However, sexual selection also plays an important role in hermaphrodites and has led to the evolution of the love dart in several species of land snails. In the foregoing chapter, I suggested that the mucus of the dart can serve to manipulate the mating partner by influencing the storage of donated sperm, and that this manipulation would be important in the competition for the fertilisation of eggs. However, the introduction of a bioactive substance by the dart could also be explained as an honest signal for mate choice. By applying evolutionary game theory, I attempt to tease apart these two alternative hypotheses.

This chapter has been submitted for publication with R. Chase as a co-author.

Abstract

The sexual conflict of interest between male and female reproductive goals can lead to an arms race in which intersexual selection and intrasexual selection become difficult to identify. In several simultaneous hermaphroditic land snails sexual selection has led to the evolution of the so-called love dart. The question central to this article is whether the dart has evolved as an honest signal for mate choice or as an instrument of mate manipulation. By applying evolutionary game theory we attempt to tease apart these two alternative hypotheses. The resulting game of darts predicts different evolutionarily stable strategies for the mate choice and the mate manipulation hypotheses. For mate choice, dart shooting is predicted to occur in every mating encounter. For mate manipulation, dart shooting is predicted to be absent at times Experiments revealed that snails do not always shoot their darts. We conclude that the dart is used as an instrument to manipulate the partner's reproductive system solely for the benefit of the shooter's reproductive success. Because such manipulation imposes a cost on reproduction in the recipient (who is also a dart shooter), sexual conflict in simultaneous hermaphrodites is demonstrated.

Introduction

Sexual selection arises from differential mating success, and it explains the evolution of secondary sexual characteristics (Darwin, 1871). Sperm competition is one form of intrasexual selection and can result in the manipulation of female reproduction to the advantage of the male. Intersexual selection, on the other hand, allows for (cryptic) mate choice on certain traits before, during and after copulation. These selection processes can potentially cause a conflict of interest (Stockley, 1997), and an arms race can develop between the sexes in which it becomes difficult to distinguish intra- and intersexual selection (Birkhead & Møller, 1993).

A behavioural oddity that remains to be fully explained by sexual selection theory is the dart shooting observed in several species of simultaneous hermaphroditic land snails. Like many sexual characteristics, the shooting of the so-called love dart forms part of an elaborate courtship behaviour. Near the end of courtship, when the animals are in close body contact, the calcareous dart is shot by a forceful eversion of the dart sac. The dart usually pierces through the skin of the mating partner and often stays lodged there (Adamo & Chase, 1988). After having shot its dart the snail everts its penis and attempts intromission. Upon successful simultaneous intromission, copulation begins and spermatophores are formed; the spermatophores are reciprocally transferred in the following hours (Adamo & Chase, 1988). Snails usually mate several times during a mating season before laying eggs, and received sperm can be stored in the spermatheca for more then a year (Lind, 1973).

The love dart is produced in the dart sac from calcium carbonate, which is transformed into the crystal aragonite (Hunt, 1979; Tompa, 1980). Upon being shot it is covered with mucus from the paired digitiform glands (Chung, 1986a; Adamo & Chase, 1990). We showed that the dart does not serve as a nuptial gift of calcium (Koene & Chase, 1998a), and that the mucus covering the dart affects the female reproductive system (Koene & Chase, 1998b). In the latter *in vitro* experiments we found that the mucus has the effect of closing off the route to the sperm-digesting bursa copulatrix and, by increasing peristalsis, probably also facilitates the uptake of the spermatophore. In *Helix aspersa* the sperm are densely packed in the spermatophore, from which they must escape once it is transferred into the partner's bursa tract diverticulum. Unlike some other

mating systems (Eberhard & Cordero, 1995), no factor in the semen comes into direct contact with the sperm-transporting female organs or the blood, therefore neither the spermatophore nor its contents are likely to influence sperm storage. However, the mucus of a penetrating dart, which enters the blood before the spermatophore is donated (Adamo & Chase, 1990), might have this function. We therefore suggested that the mucus can increase the reproductive success of the shooter (Koene & Chase, 1998b).

This idea, that dart shooting benefits the shooter by manipulating the storage of its sperm for fertilisation of the partner's eggs, the *mate manipulation* hypothesis, is not the only possible explanation for dart shooting. *Mate choice* could provide an alternative explanation of our recent findings (Eberhard, 1996). The receiving individual might use dart receipt as a selection criterion for the donated sperm. Depending on the quality of dart shooting, and thus on the amount of mucus introduced into the blood, a given quantity of sperm might be stored by the recipient. The effect of the mucus on the female reproductive system, as reported by us, would then reflect the physiological basis of the choice that the recipient makes.

Both the mate manipulation hypothesis and the mate choice hypothesis predict an effect on the paternity of the offspring. This prediction was recently tested by Landolfa, Green & Chase (unpublished data) who found that snails that shot their darts deep into the recipient fertilised a larger proportion of the recipient's eggs than less successful shooters. Here, this effect on the shooter's reproductive fitness is assumed. The question we address is how dart shooting evolved.

We begin by setting forth the rationales for the mate choice and mate manipulation hypotheses. This is followed by a brief description of evolutionary game theory, which we then apply to dart shooting. The resulting game of darts makes different testable predictions for the two alternative hypotheses. On the one hand, for mate choice by the recipient, snails are expected to always shoot a dart. On the other hand, for mate manipulation by the shooter, individuals may sometimes not shoot a dart. Our experimental results show that snails do not always shoot their darts. This leads us to conclude that dart shooting is an act of mate manipulation to increase the shooter's reproductive success. Throughout this paper we occasionally refer to separate gender functions, even though the snails themselves are simultaneous hermaphrodites.

Mate choice

Darwin (1871) proposed male-male competition and female preference for particular male features to account for secondary sexual characteristics. This idea was further developed by Fisher's runaway selection theory (1930) based on the assumption that there exists a sexual preference on an arbitrary trait in at least one sex, and that such a preference confers a reproductive advantage. The preference causes a runaway process in which the preferred trait and the preference become exaggerated, leading to the development of extreme traits like the peacock's tail. More recently the "good genes" hypothesis, also known as the handicap principle (Zahavi, 1975, 1977, 1991), has been suggested as an alternative (Andersson, 1994). In this case the preferred feature, on which the choice is based, is costly. Only individuals able to show or develop the trait despite this cost demonstrate their superior quality (an epistatic or "Zahavian" handicap). This situation is stable because the investment represented by the costly feature is compensated for by the gain in reproductive success (Zahavi, 1975; Grafen, 1990). Alternatively, a revealing handicap can show the condition of the signaller. For example, the full expression of a feature may depend on the health of the animal (Hamilton & Zuk 1982; Maynard Smith, 1985). Most known examples comprise female choice but cases of male choice are also known (e.g. Michiels & Streng, 1998); therefore, throughout the rest of this paper we use the neutral term, mate choice.

The choice for (the sperm of) a specific type of mating partner can be made before, during or after copulation. Recent evidence for post-copulatory, or cryptic, mate choice comes from the sand lizard *Lacerta agilis* (Linnaeus). In this species females mate with any available male but those males that are least related to the female sire more offspring than closely related males (Olsson *et al.*, 1996). A second example is the compound ascidian *Diplosoma listerianum* (Milne Edwards) in which ovarian mechanisms prevent self-sperm and sperm from some genetically similar clones from reaching the eggs (Bishop, Jones & Noble, 1996a,b).

Theoretically, female reproductive structures can play an important role in sperm selection (Hellriegel & Ward, 1998). Post-copulatory sperm selection can be enabled by a complex female reproductive system, such as exists in *Helix aspersa*. The spermatophore is received in the bursa tract diverticulum, which is part of the sperm-digestive bursa

complex. Sperm have to escape from the spermatophore and travel a considerable distance through the female tract in order to reach the spermatheca for storage. For *Helix aspersa* it has been found that approximately 0.05% of the donated sperm reach the spermatheca (D. Rogers, unpublished data). For *Helix pomatia* (Linnaeus) this number was estimated to be approximately 0.1% (Lind, 1973), while Haase & Baur (1995) found in *Arianta arbustorum* (Linnaeus) that some matings resulted in zero sperm stored. Sperm that do not escape fast enough are transported into the bursa copulatrix and digested (Tompa, 1984; J.M. Koene, unpublished observations).

The mucus of the love dart could be the basis for a choice made by the dart recipient. The choice could depend on the quality and quantity of the mucus transferred into the recipient and/or on the recipient's sensitivity to the active substance, resulting in more or less sperm reaching the spermatheca. According to this mate choice hypothesis both the recipient and the shooter experience an ultimate reproductive benefit from dart receipt and dart shooting, respectively. For example, the recipient's function can benefit from the opportunity to choose the sperm from the best of several partners and it has control over the fertilisation process. The shooter can benefit because its sperm is chosen based on the shooting. The cost of dart shooting is compensated for by the benefit derived from the sperm being chosen (Zahavi, 1975; Grafen, 1990). Alternatively, the quality of the mucus of the dart may reveal the presence of parasites in the shooter's reproductive tract.

Mate manipulation

According to the alternative hypothesis, the dart serves to increase the shooter's reproductive success (Adamo & Chase, 1996). Mate manipulation might influence the recipient's behaviour or endocrinology during courtship or thereafter. For example, in the housefly *Musca domestica* (Linnaeus) the male transfers a seminal factor into the female vaginal pouch. This factor enters the blood through the wall of the pouch and decreases the receptivity of the female to other males (Riemann, Moen & Thorson, 1967; Leopold *et al.*, 1971). Often such mechanisms are "sensory traps" in which the male function takes advantage of female traits evolved under natural selection (Eberhard, 1998). The mucus of the dart might act upon such a pre-existing sensitivity, for example, if the mucus were to contain a substance similar or identical to one already used in the female reproductive

endocrinology (Adamo & Chase, 1996). We have referred to such a substance as an allohormone, i.e. a hormone-like substance introduced by a conspecific (Koene & Chase, 1998b). The identity of the bioactive substance in the mucus is currently under investigation (Fortier, Koene, Nagle, Painter & Chase), but the finding that pedal gland extracts sometimes evoke the mucus effect (Koene & Chase, 1998b) suggests that the substance might be a general component of mucus. The pedal gland is the source of the mucus secreted by the foot as an aid in locomotion, and its secretory product is species specific (Chase *et al.*, 1978).

In *Drosophila melanogaster* (Meigen) a substance is transferred with the semen during copulation that augments the male's reproductive success (Fowler & Partridge, 1989; Chapman *et al.*, 1995; Rice, 1996). Males transferring this factor are more successful both in remating females and in decreasing the remating of females with competing males. However, females suffer a decrease in reproductive fitness through increased mortality due to the toxicity of the substance in the semen. This female cost was only uncovered when the females were prevented from co-evolving, because normally the female system co-evolves to counteract the negative effect of the semen. This experiment clearly demonstrates the conflict of interest between males and females (Stockley, 1997). Such a conflict can also exist between the male and female functions in hermaphrodites (Charnov, 1979) and might be the reason for the bizarre penis fencing in the hermaphroditic flatworm *Pseudoceros bifurcus* (Prudhoe) (Michiels & Newman, 1998). In these worms the penis is used to pierce the skin of the partner to deliver a hypodermic injection of sperm. Similarly, in the leech *Placobdella parasitica* (Say) a spermatophore is deposited on the skin of the partner which then digests the skin by enzymatic action; the spermatophore then contracts, causing the sperm to be injected into the body cavity (Myers, 1938). In the latter two examples high mating costs are avoided and the sperm donor gains direct access to eggs (Michiels, 1998). The sperm recipient, however, experiences the cost of wound healing and, especially, the partial loss of control over fertilisation (Eberhard, 1996).

Does the dart decrease the recipient's reproductive success through a partial loss of her control over fertilisation? To answer this question we first need to know how snails can optimise their reproductive fitness. Females of gonochoristic species select sperm

(cryptically) in different ways and on different levels (Eberhard, 1998), and in many cases they select sperm from multiple mates to fertilise eggs (e.g. Chen & Baur, 1993). For instance, genetic diversity can be advantageous in a variable environment since there is a higher chance that at least some of the offspring will survive. Diversity in the offspring also reduces the level of competition and inbreeding among the siblings. The heterozygosity theory (Brown, 1997) and histocompatibility (Brown & Eklund, 1994) can also explain why multiple matings would provide genetic benefits. Experimental evidence that polyandry increases female reproductive success through increased offspring survival comes from the adder *Vipera berus* (Linnaeus) (Madsen *et al.*, 1992) and the harlequin-beetle-riding pseudoscorpion *Cordylochernes scorpioides* (Linnaeus) (Zeh, 1997). Thus, by taking over part of the control over fertilisation, the dart may decrease the recipient's female reproductive success. Alternatively, the dart could increase the female reproductive success of the recipient, but reduce the male reproductive success of the recipient to such an extend that the overall reproductive success is lowered (Greeff & Michiels, 1999)

The promiscuity of snails and the presence of several tubules in the spermatheca suggest that sperm from several partners can be stored (*Helix aspersa*: D. Rogers, unpublished data; *Helix pomatia*: Lind, 1973; *Arianta arbustorum*: Haase & Baur, 1995). Also, the complex morphology of the sperm receiving organs in snails provides ample opportunity for cryptic sperm selection by the recipient. Thus, the recipient might have firm control over paternity. The dart could serve to change the selection criteria to benefit the shooter's reproductive success. This is supported by the recent demonstration of the positive effect of dart shooting on paternity (Landolfa, Green & Chase, unpublished data). For the mate manipulation hypothesis, the dart would then act against the recipient's sperm selection rules, causing a partial loss of control over fertilisation. The above suggests that if the dart were an instrument of mate manipulation it could impose a cost on the recipient's reproduction. This could lead to so-called chase-away selection, in which the manipulation (based on a pre-existing sensory bias) causes counteradaptive co-evolution for the avoidance of the manipulation (Holland & Rice, 1998).

The relevance of evolutionary game theory

It is not easy to determine whether any particular behavioural phenomenon is due to mate manipulation or mate choice. Indeed, this has become one of the major challenges of sexual selection theory (Birkhead & Møller, 1993). Moreover, in simultaneous hermaphrodites both processes are at work in each individual. The evolutionary game theory analyses behaviour from an economic point of view, comparing the costs and benefits of behaving in a certain way. Thus, even without detailed genetic knowledge the evolutionary game theory can make predictions about the evolution of a certain phenotype (Marrow, Johnstone & Hurst, 1996). Since Maynard Smith's description (1982), the theory has been applied successfully to evolutionary questions across a wide range of animal behaviours (Dugatkin & Reeve, 1998). An excellent example is the dung fly *Scathophaga stercoraria* (Linnaeus) (often *Scatophaga*). In this species, smaller males have a reduced rate of displacing sperm from the female sperm store, and they are less likely to take over females from other males (Parker, 1970). Based on these facts the game theory predicted that small males should copulate for longer durations than large males, and measurements of copula duration bore out the prediction (Parker & Simmons, 1994). In the side-blotched lizard *Uta stansburiana* (Baird & Girard) the existence and population dynamics of three different morphs conforms to the rock-paper-scissors game (Sinervo & Lively 1996; Maynard Smith, 1996). The three different morphs have different mating strategies: defending a large territory, defending a small territory or defending no territory (used by "sneakers"). Animals with a large territory are unable to defend it against "sneakers" that mate with the females on their territory. In turn, "sneakers" are defeated by males with small territories and one female, who can prevent the female from mating with "sneakers". This mate guarding strategy is again defeated by the large territory strategy, resulting in a dynamic cycle.

An evolutionary game can be defined as follows (Maynard Smith, 1982; Riechert & Hammerstein, 1983). The *players* of the game are individuals that may each express one of several behaviours in a given situation (e.g. a mating encounter). These different behaviours are referred to as the different *strategies* that the individual can adopt. A played strategy will give the players a certain *payoff*, either positive or negative. Payoff is expressed in units that are appropriate to the game (in the example of a mating encounter

the payoff can be a change in reproductive success, i.e. the number of sired offspring). Usually, the received payoff for a player depends both on his/her own strategy and that of the opponent. The relationships among payoffs can be expressed in a *payoff function*.

When an individual expresses one of the behaviours it is said that the strategy is "played" to maximise its payoff. This anthropomorphic description stems from classical game theory and implies that an individual makes a conscious decision about his/her strategy; however, this is not the case in evolutionary games. Maximisation of the payoff for individuals in an evolutionary game is "decided" by selection, either natural or sexual. The strategy that is selected is the one with the highest payoff.

The standard way to illustrate the game is to create a *payoff matrix* (Figure 1). In our matrix the players (A and B) have two ways to behave, resulting in four possible outcomes. Each cell of the matrix corresponds to one of the four outcomes. The *expected payoffs* (E) for the behavioural strategies (SHOOT and NOT SHOOT) are shown in the cells. On the left of each cell is the payoff for player A; on the right of each cell is the payoff for player B. For example, E(SHOOT,SHOOT) is the expected payoff received by a player playing the SHOOT strategy against another player also playing the SHOOT strategy [E(SHOOT,SHOOT) = S+R in Figure 1]. The payoffs represent the change in fitness from a *basic fitness* (W_0). Selection favours a maximisation of the payoff leading to an *evolutionarily stable strategy*, or ESS (Maynard Smith & Price, 1973). If one of the strategies always assures maximisation then this *pure strategy* will be adopted as the ESS. This means that there will be strong selection on individuals who express only this behaviour, leading to the disappearance of individuals expressing the behaviours with lesser payoffs. A second possibility is that different behaviours may be beneficial depending on the circumstances. This latter situation results in a *mixed strategy* as the ESS, in which individuals can play both behaviours, one with probability p and the other with probability $1-p$. In such a mixed strategy the played strategies are usually condition dependent (Gross, 1996).

For the game defined above we can calculate the fitnesses, W, for the behavioural strategies SHOOT and NOT SHOOT:

1. $W_{SHOOT} = W_0 + (1-p)$ E(SHOOT,SHOOT) $+ p$ E(SHOOT,NOT SHOOT)
2. $W_{NOT\ SHOOT} = W_0 + (1-p)$ E(NOT SHOOT,SHOOT) $+ p$ E(NOT SHOOT,NOT SHOOT)

In equation 1 the term $(1-p)$ E(SHOOT,SHOOT) represents the chance that a SHOOT-player will meet another SHOOT-player multiplied by the payoff received in such a situation; p E(SHOOT,NOT SHOOT) represents the chance of a SHOOT-player meeting a NOT SHOOT-player multiplied by the payoff for the SHOOT-player in this encounter. The fitness for strategy SHOOT (W_{SHOOT}) is composed of the basic fitness (W_0) plus the two terms explained above. The equations are taken from Maynard Smith (1982) and are used here only to illustrate how the game works mathematically. Applying the evolutionary game theory to dart shooting leads to different ESS predictions for the alternative hypotheses presented earlier.

A game of darts for snails

The description above has already prepared the way for the application of the evolutionary game theory to dart shooting. Obviously, the SHOOT and NOT SHOOT strategies represent "shooting a dart" and "not shooting a dart", respectively (Figure 1). The expected payoffs - S, R and S+R - require further explanation.

We define S as the overall gain of shooting a dart into the partner, which includes the costs (S_{cost}) and benefits ($S_{benefit}$) of shooting. The benefits can be for the male function and/or the female function. The male function can benefit from the dart if it manipulates the partner to increase the own fertilisation success (Koene & Chase, 1998b) and/or if it serves as a quality signal to increase the fertilisation chances of the own sperm. The female function can benefit if the dart increases either the chance of receiving sperm from the partner or the quantity of received sperm. This is obviously beneficial for the fertilisation of eggs, but possibly also affects the diversity of the offspring and allows for sperm digestion (Greeff & Michiels, 1999). The costs of producing and shooting the dart are assumed to be paid by the whole hermaphroditic individual (because it is not proven that one sexual function benefits exclusively). We want to explain why dart

shooting occurs, therefore the case of highest relevance is $S > 0$; however, $S < 0$ is also possible (see below).

Similarly, we define R as the net gain of dart receipt, which includes the possible costs (R_{cost}) and benefits ($R_{benefit}$) of receiving a dart. Again, the benefits can be separated into male and female components. The female function can benefit from the dart because a manipulating partner will sire offspring that are also manipulative or because dart shooting indicates other genetic or phenotypic qualities that allow for mate choice. Also, since six days are required for a dart to regenerate, a snail that shoots a dart has not mated for at least six days; by choosing to copulate with such a snail, the female function may be able to choose a mate who will transfer a maximum amount of sperm. The male function can also benefit if the dart indicates a longer inter-mating interval, in this case because less sperm from competing males will be present. The costs of wound healing and possible infection, as well as the cost of being manipulated are paid by the hermaphroditic animal as a whole (although manipulation might exclusively affect one sexual role).

Mate choice involves the benefit component of dart receipt ($R_{benefit}$) and the benefit component of dart shooting ($S_{benefit}$), and mate manipulation comprises the cost component of dart receipt (R_{cost}) and the benefit component of shooting a dart ($S_{benefit}$). Thus, if mate manipulation is at work, the cost of receiving (R_{cost}) will be larger than the benefit of receiving ($R_{benefit}$), thus $R < 0$; and the benefit of shooting ($S_{benefit}$) is larger than the cost (S_{cost}), hence $S > 0$ (but see below). Likewise, if mate choice applies $R_{benefit} > R_{cost}$, thus $R > 0$; and $S_{benefit} > S_{cost}$, hence $S > 0$. For simplicity we assume, for now, that when an individual shoots a dart its partner will receive it. In the case of an animal that both shoots and receives a dart, the payoffs add up, resulting in S+R [=E(SHOOT,SHOOT)]. We are looking at the effect of dart shooting on reproductive fitness, so we assume that when neither player shoots a dart the payoff is zero (0).

Again, we can calculate the reproductive fitness for both behaviours using equations 1 and 2, but now filling in the expected payoffs from the matrix:

3. $W_{SHOOT} = W_0 + (1-p)(S+R) + p\,S$
$= W_0 + S + (1-p)\,R$

4. $W_{NOT\,SHOOT} = W_0 + (1-p)\,R + p\,0$
$= W_0 + (1-p)\,R$

Here p represents the probability that an individual does not shoot a dart. Making predictions from these equations is rather simple because the only difference between them is the presence of payoff S in equation 3. To make such predictions we must assume that the dart shooting system has reached an ESS, which is a reasonable assumption (Maynard Smith, 1982). By applying the possible payoff functions to equations 3 and 4 we find three different predictions: NOT SHOOT as a pure strategy, SHOOT as a pure strategy, or a mixed strategy of SHOOT and NOT SHOOT (Table 1).

For mate choice (Table 1: prediction 1), where there is a net benefit for shooting as well as for receiving, the game of darts predicts that strategy SHOOT is the ESS because this gives the greatest fitness (W_{SHOOT}). Thus, all animals will perform dart shooting during each mating encounter, corresponding to the left upper cell in Figure 1. The implied evolutionary process can be described either by runaway selection or by the "good genes" hypothesis: Snails prefer shooters, hence, there will be strong selection on individuals to shoot darts. Possibly, snails might even evolve to facilitate the receipt of a dart. Potentially, there is a conflict of interest because the shooting individual might be better off without costly dart shooting; however, dart shooting is imposed because of the mate preference. For the recipient, not-shooting represents a low quality partner, meaning that the sperm of not-shooters will be less favoured. Therefore, snails that do not shoot their dart will either be rejected as mating partners or have all their sperm digested (if mating does take place). Because fertilisation by shooters is preferred, sexual selection is expected to result in an ESS in which all individuals shoot a dart during each mating. There can still be some variation in dart shooting just as there is variation in plumage

brightness, tail length and song features in birds. Also, in the case of a Fisherian process dart shooters are selected because of the preference for receiving a dart.

For the mate manipulation hypothesis there are several ESS possibilities depending on the payoff function. There is a conflict of interest between the players because the shooter experiences a positive payoff ($S > 0$) while the recipient receives a negative payoff ($R < 0$); thus each individual will prefer to shoot and not receive. Obviously, it is not possible for all individuals to only shoot and not receive, hence the conflict. This conflict can be resolved in different ways as shown in Table 1 by predictions 2 ,3 and 4. The first two predict pure strategies while the last leads to a mixed strategy.

For prediction 2 in Table 1 the absolute value of the benefit for shooting is smaller than the absolute value of the cost of receiving times the chance of receiving, i.e. $|S| < |(1-p)R|$. As a result the term $S + (1-p)R$ in equation 3 is smaller than zero, and the fitness for shooting becomes lower than the basic fitness, i.e. $W_{SHOOT} < W_0$. Because the fitnesses for shooting and not-shooting are both lower than the basic fitness, the ESS predicted by the game is the pure strategy NOT SHOOT. This prediction is obviously only a theoretical one because otherwise dart shooting would not be observed. In the payoff function for prediction 3 (Table 1) the absolute value of the benefit of shooting is larger than the absolute value of the cost of receiving times the chance of receiving, i.e. $|S| > |(1-p)R|$. In this case the term $S + (1-p)R$ in equation 3 is larger than zero and, because the fitness for shooting is highest ($W_{NOT\ SHOOT} < W_0 < W_{SHOOT}$), the predicted ESS is the pure strategy SHOOT.

A mixed strategy can also be predicted for the mate manipulation hypothesis (Table 1: prediction 4). Here, the payoff for shooting, S, and the payoff for receiving, R, may be variable and could even change in sign, resulting in W_{SHOOT} and $W_{NOT\ SHOOT}$ sometimes being larger and sometimes being smaller than W_0. Such a mixed strategy would be what Gross (1996) characterised as condition dependent, meaning that depending on the condition of the individual either of the behaviours is expressed. When a snail is driven by its male reproductive interest it benefits from shooting a dart ($S > 0$), because its sperm should then survive better in the recipient ($S_{benefit} > S_{cost}$). If the same snail is a dart recipient, it experiences a cost because its reproductive function is being

manipulated ($R_{cost} > R_{benefit}$). This predicts that animals prefer mating with not-shooters and may actively avoid receipt of a dart. However, one can imagine that when an animal has not mated for a long time and has little or no allosperm stored it should be primarily interested in sperm receipt ($R > 0$). This individual would not shoot a dart because the fate of the sperm it donates is less important, and dart shooting costs could be saved and used to invest in egg laying, meaning that shooting a dart has become costly ($S_{cost} > S_{benefit}$). At the same time, receiving a dart facilitates sperm transport to the storage site, meaning that dart receipt has become beneficial for such an individual ($R_{benefit} > R_{cost}$).

We can now summarise the prediction from the evolutionary game of darts for the mate choice and the mate manipulation hypotheses. For the mate choice hypothesis, in which a recipient would not choose sperm from a not-shooter, the predicted ESS is the pure strategy SHOOT. On the contrary, for mate manipulation, dart shooting is not a prerequisite for the recipient to accept sperm. In this case, the game of darts predicts that, depending on the payoff function, the ESS can be either a pure or a mixed strategy. Thus, the evolutionary game of darts predicts a possible difference in the ESS of dart shooting when based on either mate choice or mate manipulation.

To shoot or not to shoot, that is the question

For the mate choice hypothesis, the game of darts predicts a strong selective pressure in favour of dart shooting resulting in snails always shooting a dart. A contrary prediction arises from the mate manipulation hypothesis. Here shooting and not-shooting are both possible due to the conflict of interest between the shooter's function and the recipient's function. This conflict could allow for snails to sometimes not shoot their darts. In this case, the not-shooting individual benefits from its strategy as does its partner.

The crucial question we need to answer to distinguish between mate choice and mate manipulation is whether snails always shoot a dart during courtship. It has generally been assumed that dart shooting is a mandatory component of courtship in *Helix aspersa*, and some researchers have reported that snails always shoot a dart, provided they have one to shoot (e.g. Chung, 1987). However, there have also been reports of mating snails not shooting their darts (*Helix aspersa*: Giusti & Lepri, 1980; Adamo & Chase, 1990; Koene & Chase, 1998a; *Helix lucorum* (Linnaeus): Giusti & Lepri, 1980; *Arianta*

arbustorum: Baur, Locher & Baur, 1998). Because the earlier reports give little or no quantitative information, we decided to investigate whether dart shooting is an obligatory component of mating behaviour in *Helix aspersa*.

There are several types of not-shooters. Chung (1986b) has shown that snails only produce a dart after their first mating. Snails that mate for the first time do not possess a dart and sometimes do not even evert their empty dart sac (Adamo & Chase, 1988); we will call these *virgin not-shooters*. The second type of not-shooters involves snails that have previously shot a dart. The formation of a new dart takes about six days (Tompa, 1980) and the dart is never shot before it is finished (Chung, 1987; Adamo & Chase, 1988), yet snails do sometimes copulate during this period of dart regeneration. Snails that mate while regenerating a new dart are called secondary maters (Lind, 1976), so we will refer to these as *secondary not-shooters*. The last type is snails that possess a fully formed dart but nonetheless do not shoot; we will call these *primary not-shooters*.

To confirm the existence of primary not-shooters we made detailed observations of snails during courtship and copulation. Because dart shooting is not always easy to detect, special precautions were taken to ensure that none of the dart shooting events would be missed. A detailed description of these methods can be found in our previous papers (Koene & Chase, 1998a,b). In short, animals were housed individually at 20-25° C for at least ten days before the start of the experiment. They were fed every other day and were kept moist. Consecutive mating trials were separated by two weeks of isolation. Animals did not significantly increase in size during these two week periods and were paired with different mating partners in the consecutive trials.

In previous experiments we had already found a considerable number of snails not shooting their darts (32 of 80: Koene & Chase, 1998a). In these earlier experiments we controlled for secondary not-shooters by isolating animals for at least ten days before mating experiments to allow for dart regeneration. However, we did not control for virgin not-shooters. In the current experiment, to control for virgin not-shooters, we performed dissections on snails that did not shoot to verify the presence of a dart in the dart sac. We defined a snail as not-shooting if at the time of first penial eversion it had not shot a dart, because dart shooting never occurs once penial eversions begin. In an initial group of 67

animals, not-shooters were dissected several hours after copulation. In a second group of 48 animals, not-shooting individuals were dissected before copulation.

We observed 33 not-shooters among 115 mating snails as summarised in Table 2. There were no secondary not-shooters because the snails were kept in sexual isolation for at least ten days before the start of the experiment. Virgin not-shooters (N=16) were easily recognised by the presence of a jelly-like substance in the dart sac where the dart is normally attached (Chung, 1986b). Seventeen primary not-shooters were also observed. Together with secondary not-shooters, primary and virgin not-shooters represent a significant proportion of all maters (Table 2).

There was a difference in primary not-shooters depending on whether they were dissected before or after copulation (Table 3). For primary not-shooters that were dissected before copulation (N=3) the darts were found in the dart sac still attached to the tubercle, as expected. Adamo & Chase (1988) also reported several snails that did not shoot and kept their darts in the dart sac. However, the primary not-shooters that were dissected after copulation (N=14) were found to have removed their darts from the dart sac. Of these darts 12 had been transported towards the bursa copulatrix, while the remaining two were found in the bursa tract diverticulum.

Normally, when a dart is shot it breaks loose from the tubercle and stays lodged in the skin of the recipient. In our experiments, 15 animals missed their partners and retracted the dart back into the body together with the dart sac. All these darts got detached from the tubercle. Two were found in the dart sac and two were found in the genital atrium/copulatory canal area. Three were expelled during courtship. Most importantly, six of the retracted darts were transported into the bursa tract diverticulum, while only two were found in the bursa copulatrix. This is opposite from what was found for the not-shot darts, which were mostly transported into the bursa copulatrix. Thus, darts from primary not-shooters and retracted darts seem to be handled differently. Because of this result, we are confident that the darts recovered from primary not-shooters were not retracted darts of matings in which we had failed to observe the dart shooting event. Why the primary not-shooters do not keep their dart in the dart sac is unclear but it might be because the act of copulation constrains their disposition.

Another question that follows from the evolutionary game theory is whether individual snails are consistent in shooting or not shooting a dart. To determine whether primary maters (*Helix aspersa*) always shoot their darts we observed two consecutive matings two weeks apart in a group of 29 non-virgin snails (Table 4). We found that some snails shot darts during both encounters, while others failed to shoot on one or both occasions (12 of 29).

The observations described above confirm and extend earlier reports that dart shooting is an optional component of the mating behaviour in *Helix aspersa* and related species (Giusti & Lepri, 1980; Baur, Locher & Baur, 1998). We conclude that dart shooting can be absent at times. This finding supports the prediction from the mate manipulation hypothesis that shooting and not shooting can be beneficial depending on the reproductive state of the animal.

Discussion

Studies of sexual selection face a difficult task to separate intersexual selection from intrasexual selection because both can engender the evolution of a given sexual trait (Birkhead & Møller, 1993). The evolution of the love dart in hermaphroditic snails can be explained either by intersexual selection, the mate choice hypothesis, or intrasexual selection, the mate manipulation hypothesis. The recent demonstration by Landolfa, Green & Chase (unpublished data) that differences in dart shooting success are reflected in paternity is consistent with both explanations. We have applied the evolutionary game theory to the phenomenon of dart shooting in order to distinguish these two hypotheses. For mate choice, the developed game of darts predicts that snails should always shoot a dart during mating, but we find that they do not always shoot a dart. Thus, our observations are inconsistent with the mate choice hypothesis but consistent with the game theoretical prediction of a mixed strategy for the mate manipulation hypothesis.

Why would a snail not shoot its dart in certain matings, thus missing out on an opportunity to increase its reproductive success? Snails are simultaneous hermaphrodites that have to find a balance between investment in male and female reproduction. Leonard (1992) assumed that one sexual role, the one with the most control over fertilisation, is always preferred in hermaphrodites, and that the dart's purpose is to induce the partner to donate sperm. However, Leonard's model did not take into account that dart shooting

snails are *simultaneously* reciprocal hermaphrodites that donate *and* receive sperm in every mating encounter, hence there seems no need to stimulate the partner to fulfil the male role (Adamo & Chase, 1996). Therefore, we suggest that either the male or female sexual role can be preferred in an individual hermaphrodite depending on its reproductive state, even though both sexual roles are always performed. In some cases the individual might be less interested in its male reproductive success, and more interested in female reproduction, i.e. receiving sperm. In other words, not-shooting might be beneficial at times and motivated from the female side of the animal. Given the low priority for male reproduction in this situation the individual may be better off saving the costs of the dart by giving up the opportunity to manipulate the partner; in terms of the game, giving up S ($S_{cost} > S_{benefit}$). The same individual will benefit from dart receipt because it wants to receive sperm; R has become a benefit ($R_{benefit} > R_{cost}$). Mating partners of not-shooters will profit because the cost of receiving a dart is absent (Table 1: payoff S instead of S+R). To summarise, the payoffs for shooting and receiving may be variable depending on the snail's reproductive state.

The above suggests that not-shooters secure their own female reproductive success by assuring that the allosperm store contains sperm for the fertilisation of their eggs. Securing female reproduction before investing in male reproduction provides a good explanation for the existence of virgin, primary and secondary not-shooters. Haase & Baur (1995) found that the allosperm store of *Arianta arbustorum* was in some cases depleted after the production of two egg clutches. An experimental difference might be predicted between snails that have recently laid one or more clutches of eggs and snails that have mated several times but have not laid eggs. The former should have a high probability of not-shooting since they are mostly interested in replenishing their emptied allosperm stores. The latter would be mating mainly to donate sperm, and they should shoot darts. If this prediction could be confirmed it would support the idea that the payoffs in the game of darts change with the condition of the animal.

The evolution of mate manipulative strategies occurs when the recipient of manipulation has control over the internal fertilisation process (Michiels, 1998). The complicated morphology of the snail's female reproductive system offers ample opportunity for cryptic mate choice. This mate choice could, for instance, be based on the

size of the spermatophore, as in insects (e.g. Sugawara, 1979). Mating partners could be assessed early in courtship through pheromones (Chase *et al.*, 1978) or courtship behaviours like biting the partner. Since the withdrawal responses are suppressed during mating behaviour (Balaban & Chase, 1990), the strength of withdrawal upon biting could reveal the sexual motivation of the partner as suggested by Giusti & Lepri (1980, also Giusti & Adreini, 1988). This agrees with the fact that pairs of sexually active snails often separate early in courtship - to look for a different mating partner - but rarely once a dart has been shot (J.M. Koene, personal observations). Such a choice mechanism could be costly because suppressing the withdrawal reflex can make the individual more vulnerable to predators.

By manipulating the mating partner the dart shooter gains some control over fertilisation and imposes a cost on the recipient. The cost of dart receipt, besides healing the injury and risk of infection, is caused by the deviation(s) from the snail's optimal sperm selection method. This reflects the conflict of interest between the male and female functions in hermaphrodites, and increases the selective pressure for avoiding the cost, potentially resulting in an arms race. Indirect evidence for the cost imposed on the recipient comes from experiments done in *Arianta arbustorum* (Chen & Baur, 1993). In that species, snails that were allowed to mate continuously had a higher mortality rate than snails that were only allowed to mate once or twice a year. Unfortunately, it was not tested whether the increase in mortality was due to receiving more darts or to the higher mating frequency. One way for snails to avoid the cost of dart shooting would be to actively avoid receiving a dart. Darts are shot only if the animals are in close bodily contact. By withdrawing upon receipt of the dart the animal might be able to minimise the degree of penetration and thereby the cost. This implies that animals that withdraw more, or more quickly, experience less penetration and thus a lower cost, which is a testable prediction.

If snails actively avoid receipt of a dart, as suggested for mate manipulation, this avoidance would result in variable intra-individual shooting success. The success of dart shooting comprises the degree of penetration, the force of shooting, the duration of skin penetration, etc. On the contrary, for mate choice the success of dart shooting should reliably reflect or reveal the (genetic or phenotypic) quality of the shooter (Zahavi, 1975;

Pomiankowski, 1988). This means that a high quality snail should be able to consistently pierce the partner's skin with a dart while a low quality individual may often miss under constant conditions. Hence, for mate choice we predict significant differences between individuals in average shooting success. Testing this hypothesis requires measuring the shooting success of individual snails in multiple mating encounters.

For the ultimate avoidance of the cost of dart receipt the evolution of the bursa tract diverticulum is important. Visser (1977) found that the diverticulum is a rudiment of a seminal groove transporting allosperm to the spermatheca. Schileyko (1991) proposed that the possession of a dart is an ancestral trait in the superfamily Helicoidae. If the ancestral darts have a function similar to that of *Helix*, i.e. increasing the shooter's reproductive success by inducing contractions of the seminal groove, and thus promoting sperm transport, one could imagine that the diverticulum evolved to counteract this effect. In turn, the shooter, by changing the gland secretions or dart properties, may have co-evolved to gain back some control over fertilisation. It is conceivable that the introduction of the mucus to close off the bursa tract evolved due to this arms race between the male and female functions (Chase-away selection: Holland & Rice, 1998).

If Darwin (1871) had observed dart shooting he might not have claimed that hermaphroditic invertebrates are incapable of acquiring secondary sexual characteristics due to their insufficient mental powers and the union of the sexes. The data and theory support our previously posed hypothesis that the shooting of the "love dart" is a mechanism for manipulation of the recipient's reproductive success, and not a mate choice mechanism. Interestingly, even though sperm is reciprocally exchanged the conflict of interest between the sexes apparently remains. This is due to the fact that received sperm is stored for a long period of time and the snails mate several times before eggs are fertilised, thus promoting sperm competition. Our results lead the way to establishing an arms race due to sexual conflict in simultaneous hermaphrodites, and indicate that for all mating systems one has to take into account both intersexual (mate choice) and intrasexual (mate manipulation) selection mechanisms.

Conclusions

1. Sexual selection results from the effort of individuals to maximise their reproductive success. However, female reproductive success is not necessarily maximised together with male reproductive success, and *vice versa*. This potential conflict of interest between the male and female functions can cause an arms race. Theoretically, the conflict can be extended to hermaphrodites, and we hypothesise that the love dart of several species of land snails evolved due to sexual selection.
2. One way to explain dart shooting behaviour is through mate choice. In many species the sperm recipient prefers (sperm of) a specific type of mating partner based on a set of costly traits that reflect or reveal its (genetic or phenotypic) quality (Zahavi, 1975). In the mate choice hypothesis for dart shooting, its choice is based on the partner's dart shooting.
3. In the mate manipulation hypothesis, the dart increases the chance that the shooter's sperm will reach the recipient's sperm storage organ. Storage of more of the donor's sperm makes it more likely that this sperm will fertilise the recipient's eggs. This manipulation potentially decreases the recipient's reproductive success because it loses part of the control over fertilisation.
4. Evolutionary game theory can help to tease apart the two alternative hypotheses.
5. The game of darts developed herein makes different evolutionary predictions for mate choice and mate manipulation. For mate choice, animals are predicted always to shoot their darts during a mating encounter because payoffs for shooting and receiving are positive. For mate manipulation a mix of shooting and not-shooting might be expected. In this mixed strategy the payoff function depends on whether there is a priority for male or female reproduction, which is determined by the reproductive state of the individual.
6. In experiments designed to test the predicted differences, not-shooting individuals were frequently observed.
7. Mate manipulation, and not mate choice, is supported by the experimental results. We conclude that the "love dart" serves solely to benefit the shooter, to increase its reproductive success. It usually imposes a cost on the recipient's reproductive function.
8. This paper provides evidence for the existence of a conflict of interest between the male and female functions in simultaneous hermaphrodites.

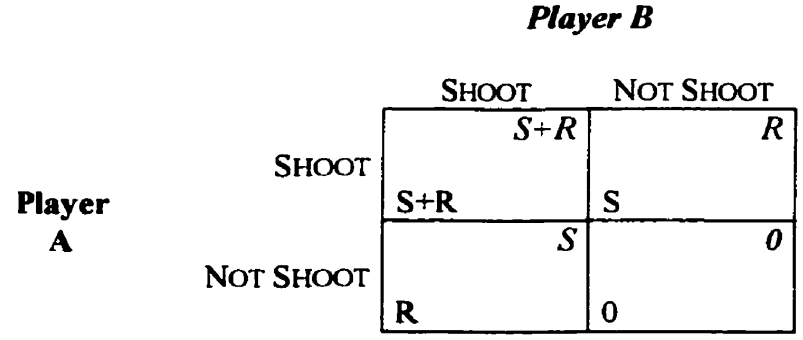

Figure 1. The evolutionary game of darts for snails. The game represents a mating encounter between two hermaphroditic snails, players A and B. Each individual has two possible behavioural strategies: SHOOT or NOT SHOOT a dart. The payoff matrix shows the expected payoffs (E) for the players A and B, respectively, on the left and the right of each cell (for clarity the payoffs for B are in *italics*). Thus, E(SHOOT,SHOOT) is the payoff received by a SHOOT-player meeting another SHOOT-player and equals S+R. S stands for the payoff for shooting a dart; R stands for the payoff for receiving a dart.

Hypothesis	Payoff function	Fitnesses	ESS
1. Mate choice	$R > 0$; $S > 0$	$W_0 < W_{\text{NOT SHOOT}} < W_{\text{SHOOT}}$	SHOOT
2. Mate manipulation	$R < 0$; $S > 0$; $S + (1-p)R < 0$	$W_{\text{NOT SHOOT}} < W_{\text{SHOOT}} < W_0$	NOT SHOOT
3. Mate manipulation	$R < 0$; $S > 0$; $S + (1-p)R > 0$	$W_{\text{NOT SHOOT}} < W_0 < W_{\text{SHOOT}}$	SHOOT
4. Mate manipulation	R and S are condition dependent	W_{SHOOT} and $W_{\text{NOT SHOOT}}$ vary, p varies	SHOOT/NOT SHOOT

Table 1. Predictions from the evolutionary game of darts. The predictions for an evolutionarily stable strategy (ESS) are found by applying the possible payoff functions for mate choice and mate manipulation to equations 3 and 4 (see text). The resulting fitnesses, W_{SHOOT} and $W_{\text{NOT SHOOT}}$, for the strategies SHOOT and NOT SHOOT, are shown relative to W_0 and to each other. From these fitness inequalities the ESS prediction is either a pure strategy (SHOOT or NOT SHOOT) or a mixed strategy (SHOOT/NOT SHOOT).

Category of snails	Number of observations	
Mating snails		115
Not-shooters	33	
Virgins	16	
Primary	17	
Shooters	82	
Successful	67	
Retracted	15	

Table 2. Observations of shooters and not-shooters. Not-shooting snails were divided into virgin not-shooters and primary not-shooters. Shooting events were divided into successful shootings in which the dart stayed lodged in the skin of the partner, and retracted shootings in which the dart returned back into the shooting animal together with the dart sac.

	Not-shot		Shot but retracted
	Dissection		Dissection
	Before copulation	After copulation	After copulation
Dart sac	3 attached	0	2 detached
Genital atrium/copulatory canal	0	0	2
Bursa tract diverticulum	0	2	6
Bursa copulatrix	0	12	2
Expelled	0	0	3

Table 3. The fate of not-shot and retracted darts. In one group of snails primary not-shooters were dissected before copulation (N=3); in another group the not-shooters were dissected after the end of copulation (N=14). The position of retracted darts (N=15) was determined by dissections performed after copulation. Darts found in the dart sac could be either attached to the tubercle or detached from it. Some retracted darts were expelled through the genital pore of the animal during courtship.

First / second dart shooting opportunity	Number of observations	Percentage of total
SHOOT / SHOOT	17	58.6
SHOOT / NOT SHOOT	5	17.2
NOT SHOOT / SHOOT	4	13.8
NOT SHOOT / NOT SHOOT	3	10.3

Table 4. Dart shooting consistency of non-virgin snails. The dart shooting behaviour of 29 animals was observed during two consecutive matings separated by two weeks of sexual isolation. SHOOT and NOT SHOOT are indicated for the first and second mating encounters.

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Chapter 5

"ALLOHORMONES": A NEW NAME FOR A CLASS OF BIOACTIVE SUBSTANCES

The finding that the love dart introduces a bioactive substance into the mating partner, and its importance for sexual selection, stimulated a search for a general term to designate such a substance. The transfer of a bioactive substance, usually during courtship or copulation, has been reported in many species but no defining term is in use. I propose the term "allohormone" for such substances to distinguish them from pheromones and hormones.

This chapter has been prepared for publication.

During courtship and copulation in many internally fertilising species, individuals have the opportunity to transfer substances that influence the behaviour or physiology of the partner. Usually such substances are male products. Because the transfer is often a covert process, many of these chemical "gifts" have been discovered only recently. Their biological effects - that range from increasing egg laying, to inhibiting remating, to affecting sperm transport or storage - are important in terms of sexual selection (Eberhard & Cordero, 1995). Nonetheless, no single defining term exists; instead, they have been referred to with diverse and descriptive names like pheromone, allocrine, seminal factor, sex peptide, ejaculate substance, male sexual product, and (male) accessory gland secretion. Therefore, there is the need for a unifying name for this important class of biologically active substances. I propose to use the term allohormone, which has already been used to describe the substance carried on the love dart of the garden snail *Helix aspersa* (Koene & Chase, 1998).

Karlson and Lücher (1959) proposed the term telemone for a class of biologically active substances similar to the ones I am concerned with here. At the time such substances had not yet been identified, although hypothetical substances were believed to diffuse into the blood from the digestive system. Since then it has become clear that many of these substances are transferred during close bodily contact, which makes the term telemone (Gk. *tele*, at a distance), meaning "arousal at a distance", counterintuitive and inadequate. Allohormone (Gk. *allos*, other; *horman*, to excite, to arouse), meaning "arousal by another", is a more appropriate term.

I define allohormones as substances that are transferred from one individual to another member of the same species and that induce a direct physiological response. Although allohormones are not necessarily limited to reproductive processes, up to now the only examples are substances transferred during mating.

Allohormones are not to be confused with allomones, a term used for interspecific pheromones. Allomones are secreted by one individual causing an individual of another species to react favourably. An example is the defence secretion of ticks against predator ants (Yoder *et al.*, 1993). Although the two terms are etymologically close, their functions are very distinct.

Unlike endocrine hormones, allohormones are transferred between individuals. Endocrine hormones are produced by specialised tissues, and transported within individuals, via the blood, to other tissues where they induce specific physiological responses. Like hormones, allohormones induce physiological responses in specific organs. On the molecular level, allohormones can be identical to or derived from hormones used in the female reproductive system. For example, female hormones, such as prostaglandins, are added to human semen by male accessory glands (Mann & Lutwik-Mann, 1981). Prostaglandins induce contractions of the smooth muscles of the uterus; thus, they potentially promote sperm transport (reviewed by Eberhard, 1996). However, regardless of their exact molecular structure and chemical properties, as long as they behave like hormones, any peptide, protein or other large molecule can function as an allohormone.

Allohormones are also fundamentally different from pheromones (Gk. *pherein*, to transfer). Pheromones have been defined as substances that are released to the outside of one animal, in minute amounts, and are detected - with specialised sensory structures - by another member of the species in which a specific reaction is induced (Karlson & Lüscher, 1959). For example, in many species of moths the males' specialised antennae, which are extremely sensitive to volatile female pheromones, allow males to locate females from as far as one hundred meters (Bradbury & Vehrencamp, 1998). Like pheromones, allohormones are secreted outside the body by specialised glands; but unlike pheromones, allohormones enter the body of a conspecific and are not detected by sensory chemoreceptors.

The foregoing indicates that the defined allohormone class includes some of the substances labelled as pheromones that are transferred during physical contact. For example, some Plethodontid (lungless) salamanders, e.g. *Desmognathus fuscus* (Arnold & Houck, 1982), possess a mental gland that produces an allohormone. During courtship the male bites or hits the skin of the female with specialised pre-maxillary teeth that are associated with this mental gland. Through the skin injury the secretion from the mental gland is introduced directly into the blood of the female. The allohormone in the secretion increases female receptivity, which results in a higher probability that she will accept the male's spermatophore (Houck & Reagan, 1990; Houck, 1998).

Some contact pheromones, on the other hand, act on specialised chemosensory receptors and should be classified as "classical" pheromones (Karlson & Lüscher, 1959). Examples of this are found in many reptiles and mammals, in which pheromones are detected by the vomeronasal organ (Døving & Trotier, 1998). For instance, in garter snakes, *Thamnophis sirtalis parietalis*, tongue flicking behaviour of the male serves to deliver a female skin secretion to the vomeronasal organ. The pheromone molecules in the skin secretion are detected by the organ's specialised chemoreceptors and evoke courtship behaviour via these receptors (Mason *et al.*, 1989).

There are many reviews that deal with male substances that influence female reproduction (e.g. Eberhard & Cordero, 1995; Eberhard, 1996), especially in insects (e.g Gillott, 1988). Here, I focus on a few examples to illustrate some of the principles of allohormones. Allohormones can act either directly on the central nervous system or on the peripheral organs of the recipient. Centrally acting allohormones can induce oviposition, as was first shown in the fruit fly *Drosophila funebris* (Baumann, 1974a,b). Likewise, they can inhibit remating of the female by acting on the central nervous system, as was shown in the house fly *Musca domestica* (Riemann *et al.*, 1967; Leopold *et al.*, 1971a,b). Allohormones can also have peripheral effects that result in oviposition or inhibition of remating. This has been reported in the barnacle *Balanus balanoides* (Barnes, Barnes & Klepal, 1977) and the adder *Vipera berus* (Andrén & Nilson, 1987), respectively. Similarly, peripherally acting allohormones have been found to induce sperm transport in the "assassin bug" *Rodnius prolixus* (Davey, 1958), and sperm storage in *Drosophila melanogaster* (Harsmann & Prout, 1994). In all these examples the allohormones are transferred during copulation together with the sperm. However, transfer can also take place before sperm is donated, i.e. during courtship. This mode of transfer is found in the previously mentioned examples of the salamander *Desmognathus fuscus* (Arnold & Houck, 1982) and the garden snail *Helix aspersa* (Koene & Chase, 1998).

All of the identified allohormones increase the donor's reproductive success, and therefore play a role in sexual selection. Because they benefit the donor's reproduction, by increasing the chance that the donated sperm will fertilise the partner's eggs, it is generally assumed that allohormones evolved through intrasexual selection (reviewed in

Birkhead & Møller, 1998). In this case an allohormone might represent a "sensory trap" used to manipulate the partner (West-Eberhard, 1979; Eberhard, 1998). However, (cryptic) mate choice can equally well cause the evolution of such traits (Cordero, 1998). An allohormone would then be a signal of quality to the partner (Zahavi, 1975), a result of a Fisherian runaway process (Fischer, 1930), or a result of chase-away selection (Holland & Rice, 1998). In order to determine whether a trait has evolved due to intrasexual or intersexual selection, experiments have to be carefully designed to tease apart the alternative hypotheses. Such experiments have recently been performed on dart shooting in *Helix aspersa*, in which the observed dart shooting behaviour was found to conform to the behaviour predicted by the intrasexual selection hypothesis of mate manipulation (Koene & Chase, submitted). Similarly, in *Drosophila melanogaster* it was shown that an allohormone transferred by the male increased his reproductive success, but at the same time it was toxic for the female. This situation causes a conflict of interest (between the sexes) resulting in a continuous arms-race between mate manipulation of the partner and avoidance of this manipulation (Chapman & Partridge, 1996; Rice, 1996; Stockley, 1997).

In recent sexual selection research many substances that act on the partner have been discovered, and these can be expected to be found in most internally fertilising species. I believe that the term allohormone will prove to be a useful name for bioactive substances that are transferred between individuals of the same species and that affect the receiver's physiology.

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Chapter 6

THE CONSERVED LOCATION OF CNS CONTROL OF MATING BEHAVIOUR IN GASTROPOD MOLLUSCS: EVIDENCE FROM A TERRESTRIAL SNAIL

The previous chapters concentrated on understanding why some terrestrial snail species shoot darts. This chapter is concerned with how dart shooting and other mating behaviours are expressed by the central nervous system. The mesocerebrum of the right cerebral ganglion has been proposed as the main control centre for mating behaviour in *Helix aspersa*. Additionally, it has been suggested that mesocerebral neurones containing the neuropeptide APGWamide or FMRFamide mediate, respectively, penial eversion or dart shooting. I test these hypotheses, which are based on *in vitro* experiments, using a neuroethological *in vivo* approach.

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Summary

We investigated the role of the right mesocerebrum in the expression of mating behaviour in the garden snail *Helix aspersa*. Using an *in vivo* stimulation and recording technique we provide evidence for both sensory and motor functions in the mesocerebral neuronal population. Some neurones were specifically sensitive to tactile stimuli delivered to the skin on the superior tentacles and around the genital pore. Electrical stimulation of the right mesocerebrum evoked genital eversion and, in combination with tactile stimulation, dart shooting and penial eversion. Genital eversions were also elicited by injections of APGWamide. During courtship, one recorded unit increased its activity only in correlation with penial eversion, while six other units increased their activity only during dart shooting. Three additional units increased their activity during both of these behaviours. Also, most of the recorded units showed increased neuronal activity during times of contact with the partner. Comparison of our results with available data from other molluscs leads us to conclude that the right anteromedial region of the cerebral ganglion is an evolutionarily conserved region of the gastropod brain specialised for the control of male mating behaviour.

Introduction

The large size of neuronal cells in gastropod molluscs has allowed many neurones of the central nervous system (CNS) to be identified as unique individuals. The identifiability of neurones has, in turn, contributed greatly to the assignment of function, and hence to the understanding of behavioural control. While the functions of the various ganglia are known in broad outline, the regional localisation of function within the ganglia is poorly understood. The present experiments were designed to identify a function for a single lobe in the brain of the common garden snail *Helix aspersa*, which is a representative of the order Stylommatophora in the subclass Pulmonata. By implication, our results also provide insights into the ganglionic organisation of two other taxa, namely the Basommatophora, represented by *Lymnaea*, and the Opisthobranchia, represented by *Aplysia*.

The cerebral ganglion of *Aplysia* is generally thought to be organised in eight cell clusters (Jahan-Parwar and Fredman, 1976). For *Lymnaea*, three lobes are recognised (De Boer *et al.*, 1996), and in *Helix* three to five lobes are recognised (Bullock and Horridge, 1965). Only in a few cases are the functions of the lobes and clusters known. Thus, the procerebrum is an olfactory lobe in *Helix* (Ratté and Chase, 1997); the caudo-dorsal cells initiate egg laying in *Lymnaea* (Ter Maat *et al.*, 1986); and the anterior lobe regulates the expression of male sexual behaviour in *Lymnaea* (De Boer *et al.*, 1997). Significantly, homologous representations of the clusters and lobes have not been identified across taxa. It has been argued, though, that neurones variously located in the right mesocerebrum of *Helix*, the right anterior lobe of *Lymnaea*, and the H-cluster of *Aplysia* are homologous based on a common expression of the neuropeptide APGWamide (Fan *et al.*, 1997). Here we present additional evidence that these neuronal populations are homologous by the criterion of function.

Helix is a simultaneous reciprocal hermaphrodite. Mating is preceded by a protracted courtship that involves repeated mutual contacts of the tentacles, lips and genitalia (Adamo and Chase, 1988). The peripheral genital structures are progressively everted through recognisable stages. After 30 to 60 minutes, the calcareous "love" dart is thrust into the skin of the mating partner (Stage 5). Recently it was shown that the dart is used to introduce a bioactive substance into the partner's blood to influence the female

reproductive organs (Koene and Chase, 1998b). After dart shooting the animals attempt to achieve simultaneous intromission (Stage 6). If successful, they enter the copulatory phase, which requires six to eight hours for complete transfer of the spermatophore.

Most of the reproductive organs are located on the right side of the animal, which is reflected in the bilateral size asymmetry of the mesocerebral lobes. The mesocerebrum is proposed to have a function in mating behaviour based on its afferent and efferent connections, studied *in vitro* (Chase, 1986; Chase and Li, 1994). It has been suggested that some neurones contain the neuropeptide FMRFamide and mediate dart shooting, while other neurones contain APGWamide and mediate penial eversion (Li and Chase, 1995).

Material and Methods

Specimens of the garden snail *Helix aspersa* Müller were kept moist at 20°C with a light:dark cycle of 16h:8h. They were fed lettuce, carrots and chalk every other day. The snails were housed in isolation for at least two weeks before being used in experiments.

A stainless steel fine wire electrode (California Fine Wire Company), diameter 25 μm, was implanted on the right mesocerebrum for electrical stimulation and extracellular recording. The Teflon coating was removed from the end of the wire, and the naked ending was bent into a small loop (diameter approximately 200 μm). The loop was then bent 90° relative to the rest of the wire (Figure 1A). The procedure was similar to that used previously in *Lymnaea stagnalis* (Hermann *et al.*, 1994; Yeoman *et al.*, 1994).

For implantation, a snail was anaesthetised with 2-3 ml of 60 mM $MgCl_2$ (pH=7.8) injected into the back of the foot just beneath the shell. The animal was pinned down with two small pins through the front of the foot, while the shell was held back with a larger pin. At the anterior-posterior position of the genital pore a 2 mm incision was made in the skin near the dorsal midline. The cut was made about 2 mm to the left of the dorsal midline to avoid the reproductive organs.

The cerebral ganglia were gently pulled out through the incision and stabilised on a plastic hook which was attached to a micro-manipulator. The overlying connective tissue was carefully freed from the right mesocerebral neurones using two pairs of fine forceps. The mesocerebrum was then dried with a weak jet of air, and the fine wire was glued in place (Instant SuperGlue, World Precision Instruments). The glue covered the

mesocerebral neurones and some of the surrounding connective tissue. The site of implantation is shown in Figure 1A. The glue was dried by air, after which some $MgCl_2$ solution was added on, and then it was airdried again until the glue turned white. The brain was then lowered back into the body cavity. A second fine wire with a naked ending was inserted into the body cavity to serve as a reference electrode.

The incision was sutured (Braun Mirafil, 0.2 mm) to close the body cavity and, especially, to hold the fine wires in position. A length of wire was left inside the animal to avoid stress on the glued junction. The pair of wires was held together with silicone elastomere glue (KwikCast, World Precision Instruments). The wires were attached to the shell, leaving enough length for the animal to fully extend and fully withdraw into its shell. Beyond the glued site the wires were held together with light bodied polyvinylsiloxane impression material (Kerr Extrude), and they were fit with Harwin plugs at the ends. The snail was then injected with 2-3 ml of saline (Prescott *et al.*, 1997) and allowed to recover. Figure 1B shows an animal after implantation. The animals were sacrificed following the experiments to verify the position of the electrodes.

Only sexually active snails were implanted with wires. To determine sexual activity, 12-24 snails, all previously isolated, were placed in a small chamber. When courting pairs formed, one snail was taken for implantation of a fine wire while the other snail was returned to an isolation chamber. The next day, after recovery of the operated animal, the two snails were put back together again. If courtship behaviour was observed, the electrical activity and the behaviour were recorded on super-VHS videotape. The electrical signal from the fine wire was fed through a DAM-80 differential amplifier (100x amplification, bandpass 3 Hz -10 kHz; World Precision Instruments) before it was stored on the hi-fi track of the video tape. Every video frame was provided with a time code (VITC, Alpermann and Velte) to synchronise the data. The electrical activity was digitised (Cambridge Electronic Design model 1401, 12 bit analog-to-digital converter) and a program using a template matching algorithm for wave form recognition (Jansen and Ter Maat, 1992) was used to reconstruct individual spike trains from the multi-neuronal recording. A group of wave forms associated with one template is referred to as a unit.

If no courtship behaviour occurred after implantation the animals were used for tactile and electrical stimulation experiments. Several skin areas were stimulated with a hand held fine plastic filament to test for mesocerebral responses. For electrical stimulation of the right mesocerebrum through the fine wire, 5 ms pulses were delivered at 2 Hz for 3-6 minutes. The voltage was set at 50% of the threshold for a single pulse to evoke a visible local skin contraction. Sometimes electrical stimulation was combined with a tactile stimulation of the genital pore.

Neuropeptides were injected into non-sexually active snails at the back of the foot just beneath the shell. The volume of the blood of *Helix aspersa* is estimated to be ca. 2.0-2.5 ml (Koene and Chase, 1998b). Each injection contained a 50 μl solution of peptide(s) dissolved in saline. A snail was injected only once with a single dose (10^{-3} to 10^{-7} M) of APGWamide (Ala-Pro-Gly-Trp-NH_2, American Peptide), FMRFamide (Phe-Met-Arg-Phe-NH_2, Peninsula), or a mixture of APGWamide and FMRFamide at equal concentrations. Saline injections were used as a control. Approximate final concentrations in the blood were 10^{-5} to 10^{-9} M. APGWamide concentrations of 10^{-6} M and higher have been shown to inhibit contraction of the penis retractor muscle in *Lymnaea stagnalis* (Croll *et al.*, 1991).

For behavioural analysis, the mating components were defined as follows. The stages of genital eversion, from 1 to 6, were scored according to Adamo and Chase (1988). The duration of the dart shooting event was the time from when the dart first began to emerge from the shooting animal to when the dart sac was again fully withdrawn. Dart receipt was taken to occur between the time when the dart hit the recipient's skin and when the shooter withdrew its dart sac. The duration of penial eversion was the time from the initial externalisation of the penis to its ensuing retraction. Simultaneous intromission was defined as intromission that was mutually successful and sustained.

The data are reported as means ± standard deviations wherever applicable. For the *in vivo* recording the correlation between spiking activity and the different behaviours was investigated by means of permutation tests as described elsewhere (Jansen *et al.*, 1997). In short, randomisation techniques were used to calculate the probability that an experimentally found relation was due to chance by sampling a large number of random

possibilities (4000 permutation runs). Experimentally obtained bin counts were compared with a 'population' of bin counts obtained from the same recording by data permutation. Because every individual bin of the experimentally obtained histogram was compared with this "population", we thus made multiple comparisons, with the number of comparisons being equal to the number of bins in the histogram. Since we wish to accept, for every comparison, an alpha error of p=0.05, the actual significance level used in each test was adjusted by dividing p by the number of bins in the histogram. A detailed description of this analytical procedure is available elsewhere (Jansen *et al.*, 1999).

Results

Tactile and electrical stimulation

Areas of the skin were stimulated with a mechanical probe while mesocerebral activity was recorded. The sensitive skin area of the mesocerebrum was determined by mapping neural responses to the body profile (Figure 2A). On the left side of the animal, sensitivity is confined to the superior tentacle, but includes the entire length of the tentacle, as well as its base. Touching the skin between the superior tentacles also evoked spiking activity. On the right side, the sensitive skin area stretches from the tentacle base to the genital pore. Responses fell sharply as the probe was moved away from the genital pore (Figure 2A). Typically, a short burst of spikes was observed when the skin was stimulated in the sensitive skin area (Figure 2B).

To test whether neurones of the right mesocerebrum can mediate part of the mating behaviour, electrical stimulation trials were performed. In most cases stimulation evoked an eversion of the genital pore (Table 1, first column). The eversion usually reached an advanced state (stage 5) within a few minutes of continuous stimulation at 2 Hz. Except for the fact that they developed faster, the observed eversions looked identical to those seen during normal courtship (Adamo and Chase, 1988). The eversions were maintained as long as the electrical stimulation continued. Once the stimulus was terminated, the genitalia retracted to their normal positions within one minute. Electrical stimulations of the right metacerebrum (also known as postcerebrum) did not evoke genital eversion (N=5).

Tactile stimulation of the everted genital pore, when combined with simultaneous electrical stimulation of the mesocerebrum, evoked penial eversion (Table 1: second column). In two cases, the combination of electrical and tactile stimulation elicited dart shooting as well as penial eversion. Tactile stimulation alone did not evoke any eversion (Table 1: third column). When an electrically stimulated snail was paired with a sexually active partner, the stimulated snail performed normal courtship and copulation behaviours (N=2; not shown in Table 1). This last result is especially striking in light of the fact that only two of 166 implanted animals courted and mated in the absence of electrical stimulation (see below).

Injection of APGWamide elicits genital eversion

The peptides APGWamide and FMRFamide have been implicated in the mating behaviour of *Helix aspersa*. Specifically, it has been suggested that APGWamide mediates penial eversion whereas FMRFamide mediates dart shooting (Li and Chase, 1995). To test this hypothesis, the peptides were injected into the blood of unoperated animals. With this method, when combined with nerve lesions, APGWamide has been shown to act peripherally on the penial complex of *Lymnaea stagnalis* (De Boer *et al.*, 1997).

APGWamide (50 μl at 10^{-5} M or greater) evoked an eversion of the genital pore identical to that seen during normal courtship behaviour (similar concentrations evoked eversion in *Lymnaea stagnalis*: De Boer *et al.*, 1997). The eversion developed quickly to stage 5, as with electrical stimulation, and it lasted 8.59 ± 2.97 min (N=24). There was no additional effect when tactile stimulation of the genital pore was combined with the APGWamide injection. Contrary to the expectation that FMRFamide mediates dart shooting, the injections of FMRFamide (50 μl at 10^{-5} M or greater) did not have any obvious effect on the animal. However, when a mixture of APGWamide and FMRFamide was injected (both dosages 25 μl at 2 x 10^{-5} M), considerably fewer animals showed genital eversion than when APGWamide alone was injected (Table 2).

Fine wire recordings during mating behaviour

The electrical signal from the right mesocerebrum was generally silent during locomotion and feeding. Although most of the operated animals remained active, and looked healthy, only two of them engaged in courtship and copulation. Unfortunately the signal-to-noise ratio recorded from one of the animals was inadequate, thus leaving just one animal for the analysis of electrical events during mating behaviour.

Figure 3 shows the first 2.3 hours of the recording during a natural sequence of courtship and mating. The top trace shows the analog multi-neuronal recording, with markings to indicate the occurrence of dart shooting, dart receipt and simultaneous intromission. The overall spiking frequency dropped sharply from 0.52 spikes/sec during courtship to 0.06 spikes/sec after simultaneous intromission. The numbered traces in Figure 3 are the reconstructed spike trains of individual units that were extracted from the original record using a spike sorting program (Jansen and Ter Maat, 1992). Only those units selected by the spike sorting program with an amplitude above the noise level were used for analysis, because spikes with smaller amplitudes are not well discriminated (Jansen and Ter Maat, 1992). The reconstructed traces indicate that most units were highly active during courtship, but soon after simultaneous intromission (SI) their activity decreased, changed, or stopped completely.

The aggregate neuronal activity in the mesocerebrum during specific times of interest is shown in Figure 4 where it is correlated with four behaviours or events that were attributable to the implanted animal: contact with the partner, dart shooting, dart receipt, and penial eversion. Dart shooting and dart receipt each occurred just once, whereas there were numerous contacts with the partner and multiple penial eversions before simultaneous intromission. Neural activity was therefore averaged for the latter two events. Pronounced increases in activity were correlated with all events except dart receipt (Figure 4C). The dart was received in the right side of the animal 5 mm behind the genital pore outside the sensitive skin area of the mesocerebral neurones (Figure 2A). Contact (Figure 4A) and dart shooting (Figure 4B) were both correlated with peaks of neural activity at the start of the behaviours. For penial eversion (Figure 4D), an initial peak of activity can be seen just prior to the start of the behaviour followed by a second peak near the end of the behaviour. To investigate the activity patterns of individual units,

these were plotted separately relative to each behavioural event. None of the units changed its activity in any significant way at the time the animal received a dart (data not shown). The changes during dart shooting, penial eversion and contact with the partner were subjected to further analysis, as described below.

To investigate the idea that dart shooting and penial eversion are separately controlled by different neurones in the mesocerebrum, spike counts during these two behaviours are plotted together for each unit in Figure 5. One unit increased its activity only during penial eversions (unit 3), while other units increased their activity only during dart shooting (units 1, 5, 7-10). Three units increased their activity during both behaviours (units 2, 4 and 6) and, for each of these, there was a statistically significant correlation of spiking activity with penial eversion. One unit was unaffected by either behaviour (unit 11). The activity of several units (units 2-4 and 6) showed the same double peak during penial eversion as seen in the aggregate records (Figure 4D). By examining the patterns of spiking activity in individual units relative to contact with the partner we found that most units increased their activity during times of contact, but only units 5 and 9 were statistically significant (Figure 6). This result is consistent with results from the sensory mapping experiment (Figure 2) and the analysis of aggregate activity (Figure 4A).

Discussion

The results presented here suggest that neurones of the right mesocerebrum play a key role in controlling the mating behaviour of *Helix aspersa*. Our data confirm that the mesocerebrum should be regarded as an integrative centre with both sensory and motor functions (Chase and Li, 1994). The demonstration that APGWamide can mediate genital eversion in *Helix* links these findings to previous studies of mating behaviour in related gastropod molluscs and leads us to conclude that the localisation of function is evolutionarily conserved in this group of animals.

During courtship, the snails' tentacles repeatedly touch and interlock (Adamo and Chase, 1988). Thus, it was expected that mesocerebral activity would be especially responsive to tactile stimulation of the tentacles, and this was the case. However, there is also a great deal of lip contact during courtship (Adamo and Chase, 1988), and the lip nerves provide strong excitatory input to the mesocerebrum (Chase, 1986). Despite this, tactile stimulation in the present experiments did not evoke neural activity in the

mesocerebrum. The neurones of the mesocerebrum have a diameter between 25 to 80 μm (Li and Chase, 1995) and the recorded area of the mesocerebrum had an approximate diameter of 200 μm. Thus, only a small sample of the total population of ca. 140 right mesocerebral neurones (Chase, 1986) was recorded with the fine wire. Possibly, sensory information from lip contacts is selectively processed by mesocerebral neurones located at sites not recorded by the implanted wire. The fact that the receipt of a dart does not seem to evoke activity in the mesocerebrum is consistent with the idea that the mechanical action of the dart is only incidental to its function (Koene and Chase, 1998a,b).

The suggested motor function for the mesocerebrum (Chase, 1986) was confirmed by electrical stimulation via the implanted wire, although penial eversion was rarely observed unless a tactile stimulus was added to the electrical stimulus (Table 1). The tactile stimulus had to be applied specifically to the everted genital pore to evoke penial eversion and, in some cases, dart shooting. Possibly, skin stimulation provides a key input to a second motor control centre, in the pedal ganglion, as discussed below. It is noteworthy that the artificially evoked instances of penial eversion were not always preceded by dart shooting. This is consistent with behavioural observations that dart shooting is occasionally omitted even in natural matings (Koene and Chase, submitted).

The injections of neuropeptides yielded some unexpected results. APGWamide evoked genital eversion but not penial eversion, while FMRFamide, proposed to be responsible for dart shooting (Li and Chase, 1995), had no overt effect. We tested whether dart shooting required a pre-established genital eversion by injecting a combination of both peptides. Again, we observed no dart shooting; moreover, we observed fewer genital eversions than with APGWamide alone. The most likely explanation for the latter result is that APGWamide and FMRFamide have opposite effects on the penial retractor muscle. Whereas APGWamide relaxes the muscle (*Lymnaea*: De Boer *et al.*, 1997), FMRFamide contracts it (*Helix*: Lehman and Greenberg, 1987). It therefore seems likely that APGWamide is responsible for everting the genitalia, while FMRFamide is responsible for retracting them after mating and possibly for holding them inside. The role of FMRFamide in dart shooting, if any, remains to be determined. Some care must be taken with the interpretation of the FMRFamide results given that its presence in the mesocerebrum of *Helix* is controversial

(Elekes and Nässel, 1990; Cottrell *et al.*, 1992; Li and Chase, 1995). Also, the injection method might not be appropriate because of the involvement of FMRFamide in other behaviours.

The fact that very few of the operated snails showed sexual activity is most likely due to the loss of sexual motivation caused by the operation. Similar effects were observed during *in vivo* experiments with *Lymnaea stagnalis* (De Boer *et al.*, 1997). While our data on the correlation between neural and behavioural activity are derived from just a single animal, this does not affect the validity of the positive results. Caution must be exercised, however, in respect to negative results.

Most of the recorded units increased their activity during dart shooting and/or penial eversion (Figure 5). The units active during both behaviours were already anticipated by earlier *in vitro* tests of motor function (Chase, 1986) as well as by immunohistochemical and anatomical findings that suggested some mesocerebral neurones may be multifunctional (Li and Chase, 1995).

Together with other evidence, our data suggest that the neural control of mating has evolved conservatively in the gastropod subclasses Opisthobranchia and Pulmonata (Figure 7). To summarise the evidence for *Helix aspersa*, the right mesocerebrum is an integrative centre for mating behaviour based on the following evidence: anatomical studies of axon projections (Chase and Li, 1994), recorded responses to peripheral stimulation (Chase, 1986 and this paper), direct electrical stimulation (this paper), and fine wire recordings during mating behaviour (this paper). Additionally, the demonstration here that the neuropeptide APGWamide can mediate genital eversion is consistent with the immunohistochemical localisation of APGWamide in the mesocerebrum (Li and Chase, 1995).

In *Lymnaea stagnalis*, a representative of the Basommatophora, backfills of the *nervus penis* demonstrate a strong projection from the anterior lobe of the right cerebral ganglion (De Boer *et al.*, 1997). Electrical recordings from the lobe, using the implanted fine wire technique, show increased activity coincident with eversion of the preputium, a part of the male copulatory apparatus (De Boer *et al.*, 1997). Eversion can be evoked by direct electrical stimulation of the lobe, and it is mediated by APGWamide, which is expressed in the lobe (Croll and Van Minnen, 1992). It is significant that the

mesocerebrum of *Helix* and the anterior lobe of *Lymnaea* are both located at the same anteromedial position in the cerebral ganglion, and both regions are larger on the right side than on the left side (Figures 7A and 7B).

In *Aplysia californica*, a representative of the Opisthobranchia, the H-cluster is also located at the anteromedial margin of the right cerebral ganglion; there is no counterpart in the left ganglion (Figure 7C). The neurones of this cluster send processes to the penial complex, via the lower labial nerve (Jahan-Parwar and Fredman, 1976). An early study found that electrical stimulation of the right cerebral ganglion causes contractions in the penial complex (Bottazzi and Enriques, 1900). Recently, it has been reported that APGWamide is strongly present in the H-cluster (Fan *et al.*, 1997), and APGWamide can evoke penial eversion in a reduced preparation (Yu and Blankenship, 1997). In summary, the evidence from *Helix*, *Lymnaea* and *Aplysia* indicates that there is an homologous group of neurones at the anteromedial margin of the right cerebral ganglion and that these neurones are responsible, at least in part, for the control of mating behaviour.

The immunohistochemical localisation of APGWamide in several additional species of gastropod molluscs suggests that the homology is robust within the class Gastropoda. In a comparative study, De Lange and Van Minnen (1998) found immunoreactivity for APGWamide in clusters of neurones at the anteromedial margin of the cerebral ganglia in the basommatophore *Bulimus truncatus*, the stylommatophores *Arion ater* and *Limax maximus*, and even in the prosobranch *Littorina littorea*.

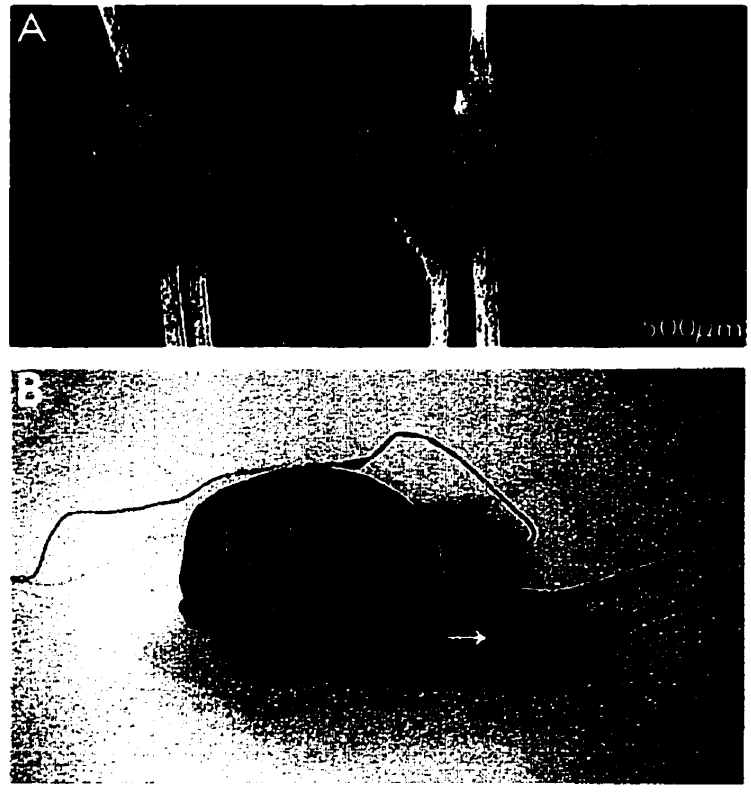
There is another cluster of neurones, located in the lateroventral region of the right pedal ganglion, which also has a role in the expression of mating behaviour. Most of these cells have projections into the penial nerve and seem to be motoneurones controlling muscles of the penial complex. Their approximate locations are shown in Figure 7 (*Helix*: Eberhardt and Wabnitz, 1979; Li and Chase, 1995; *Lymnaea*: De Boer *et al.*, 1996: *Aplysia*: Rock *et al.*, 1977). Some of the pedal motoneurones project not into the penial nerve, but into a pedal nerve which is itself a pathway to the penial complex. In *Helix*, this nerve is the NCPPD (Li and Chase, 1995); in *Lymnaea*, the NCS (Elo, 1938); in *Aplysia*, a branch of the nerve P2 (Kandel, 1979).

Although the particular function, or functions, of the pedal ganglion neurones have yet to be determined, these cells should be regarded as constituting another centre for motor control of the penial complex. Whether they operate downstream from right anteromedial cerebral ganglion neurones, or independently, is unclear, although some axons of right anteromedial neurones do terminate in the pedal ganglion (*Helix*: Li and Chase, 1995; *Lymnaea*: De Boer *et al.*, 1997: *Limax* and *Arion*: De Lange and Van Minnen, 1998). If the pedal neurones must be activated for full motor expression, and if their excitation depends on inputs additional to those from the mesocerebrum, this might explain why simple electrical stimulation of the right mesocerebrum elicits only partial genital eversion.

Our data establish the existence of an evolutionarily conserved region of the gastropod brain that is responsible for the central control of the male genitalia. The pulmonates and the opisthobranchs emerged from their prosobranch ancestors sometime in the Carboniferous period, about 350 million years ago. From then to the present, the mating behaviours evolved and differentiated. For example, *Helix* mates as a simultaneous reciprocal hermaphrodite; *Lymnaea* is a serial reciprocal hermaphrodite; *Aplysia* is a simultaneous non-reciprocal hermaphrodite. There are numerous differences in the details of courtship and copulation, including the unique dart shooting behaviour of *Helix*. It will now be interesting to investigate how the circuitry and cellular properties of sex related neurones have evolved to accommodate the different mating strategies.

[Figure on next page]

Figure 1. Implantation of the fine wire. *A.* The cerebral ganglia are shown with the connective tissue removed. A fine wire is drawn near the right mesocerebrum to indicate its size, shape and site of implantation. *B.* Photograph of a snail implanted with a fine wire. The arrow points to the partially everted genital pore.


A
500μm
B

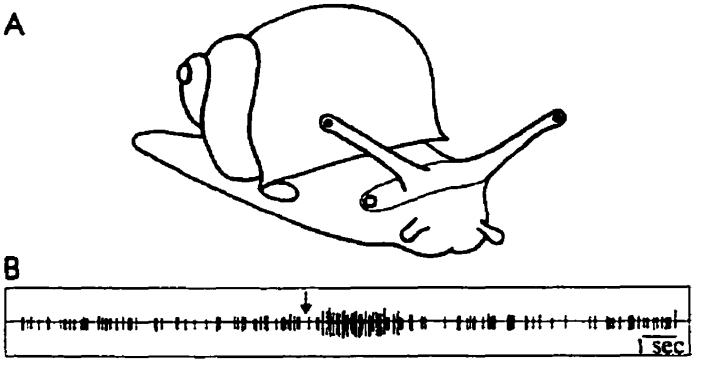


Figure 2. The *in vivo* sensitivity of mesocerebral neurones to tactile stimulation. *A.* The shaded area indicates the sensitive skin area of the mesocerebrum for stimulation with a plastic filament (N=13). *B.* A typical response to tactile stimulation (arrow), shown here for stimulation of the right tentacle. The trace is digitised and multi-unit.

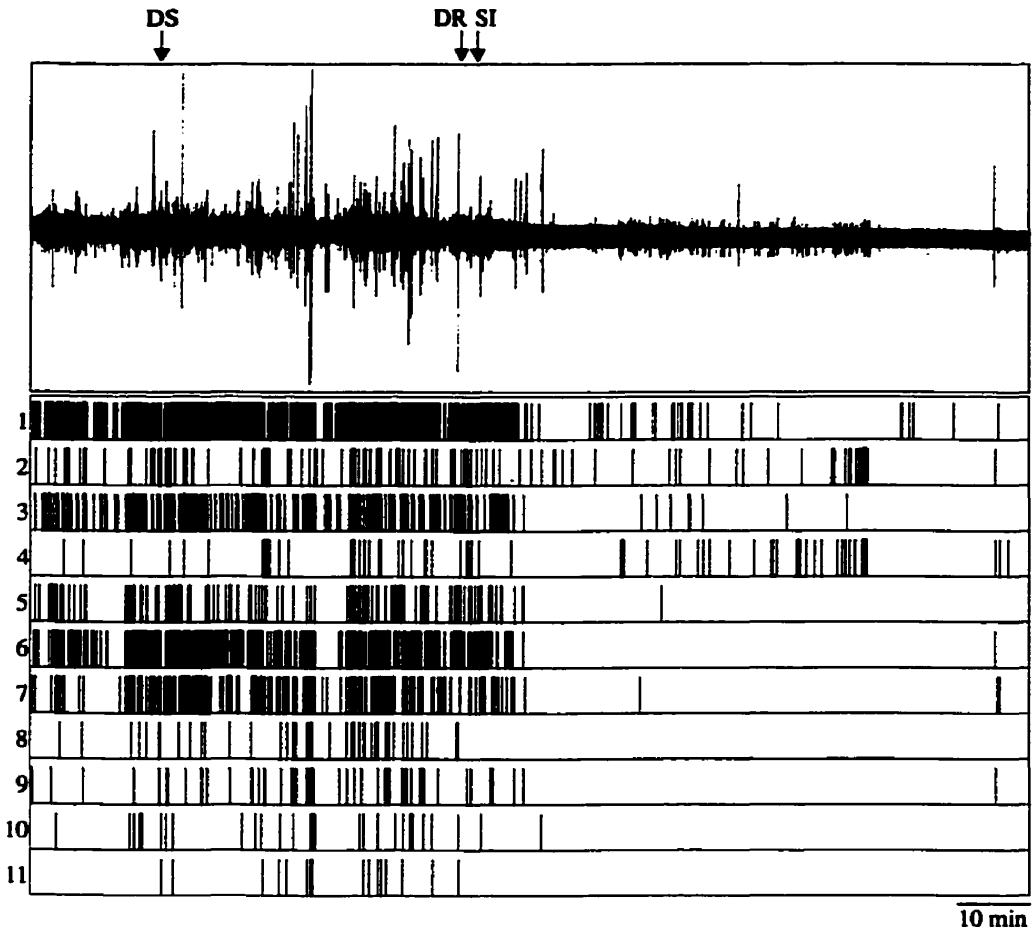


Figure 3. A recording of neural activity in the right mesocerebrum during mating behaviour. The top trace shows the analog multi-unit activity recorded before and after the transition from courtship to copulation (simultaneous reciprocal intromission). Arrows above the records indicate times for dart shooting (DS), dart receipt (DR), and simultaneous intromission (SI). The lower traces represent 11 units extracted from the multi-unit record using a spike sorting program (Jansen and Ter Maat, 1992). The units are ordered top to bottom by increasing action potential amplitude.

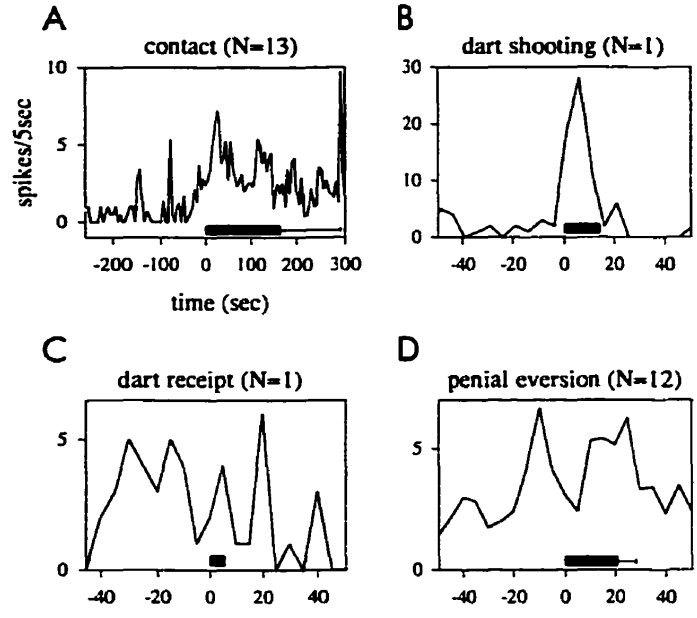


Figure 4. Aggregate spiking frequencies in relation to four different behaviours. Time zero indicates the start of each behaviour. The horizontal bars indicate the duration of the behaviour. *Dart shooting* and *dart receipt* were unique events, whereas *contact* with the partner and *penial eversion* were repeated events whose average durations ± standard deviations are indicated.

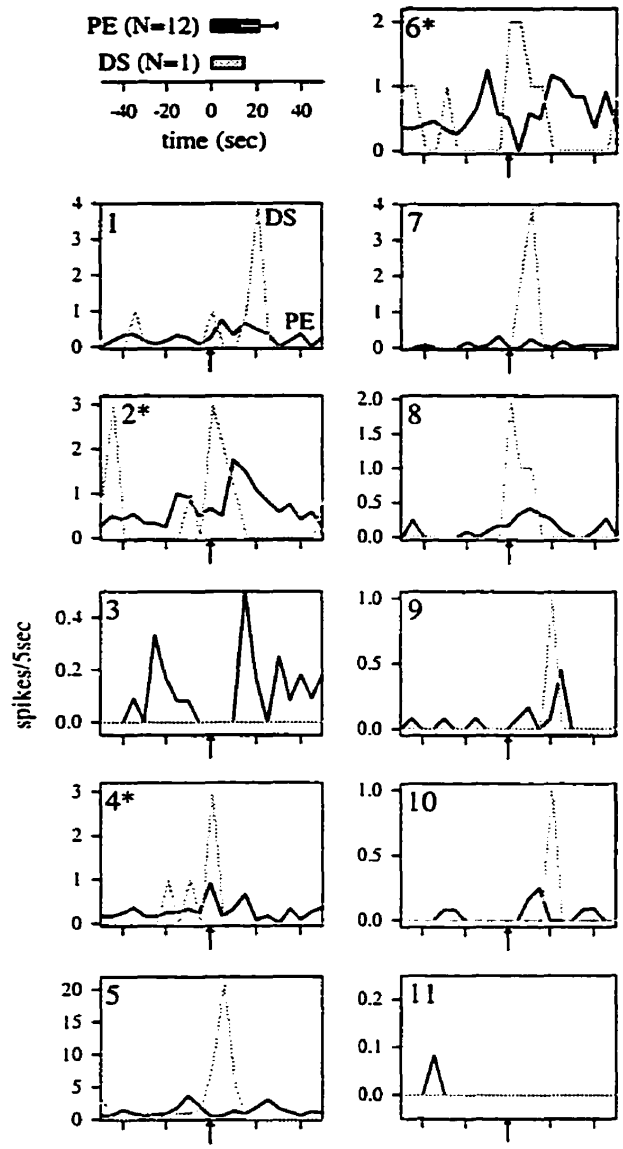


Figure 5. Comparison of spiking activity in 11 units during penial eversion and dart shooting. The timing of the behaviours relative to the spike counts is shown at the top left, where the same time scale is used as in the unit graphs. The behaviours, penial eversion (PE) and dart shooting (DS), begin at time zero (indicated by arrows in the graphs) and their durations are indicated by horizontal bars. The bar for penial eversion shows the mean duration with the standard deviation. Each graph illustrates a different unit; the unit number is indicated in the upper left corner. Spiking frequencies are shown during penial eversion (black) and dart shooting (shaded). Statistical significance of frequency changes during penial eversion is indicated by an asterisk ($p<0.05$).

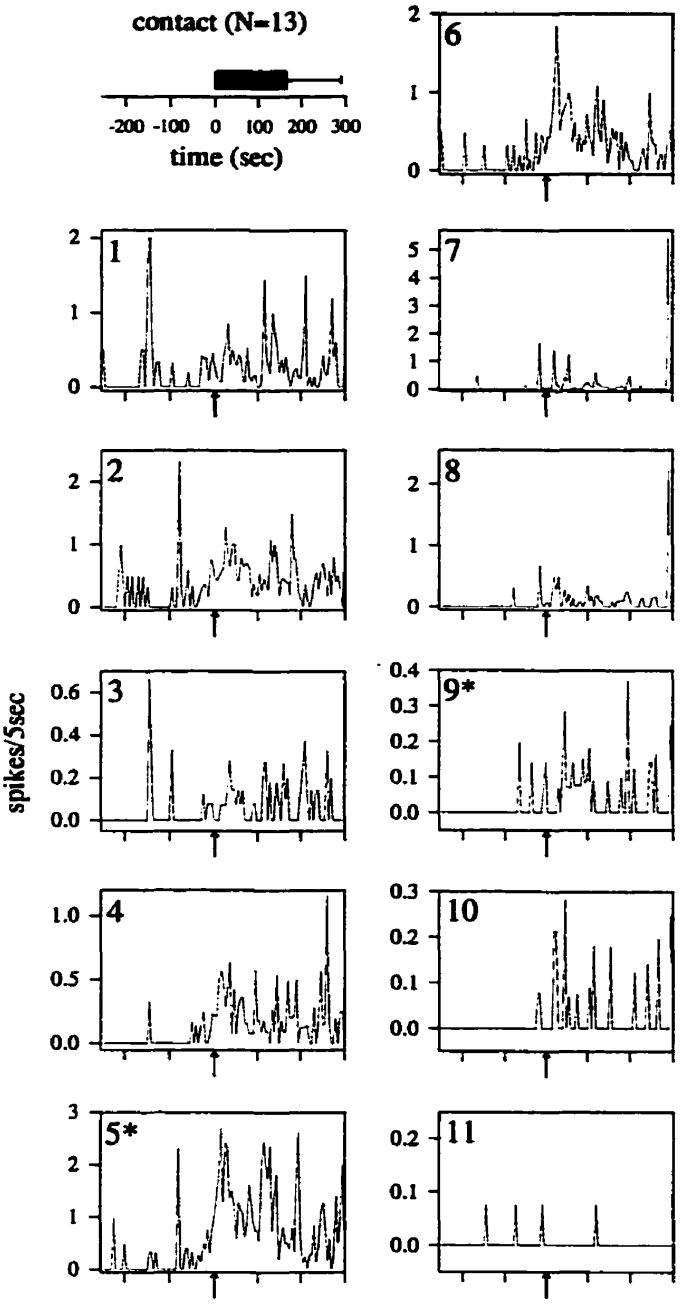


Figure 6. The spiking activity of 11 units in relation to contacts with the mating partner during courtship. See legend of Figure 5 for details of the presentation.

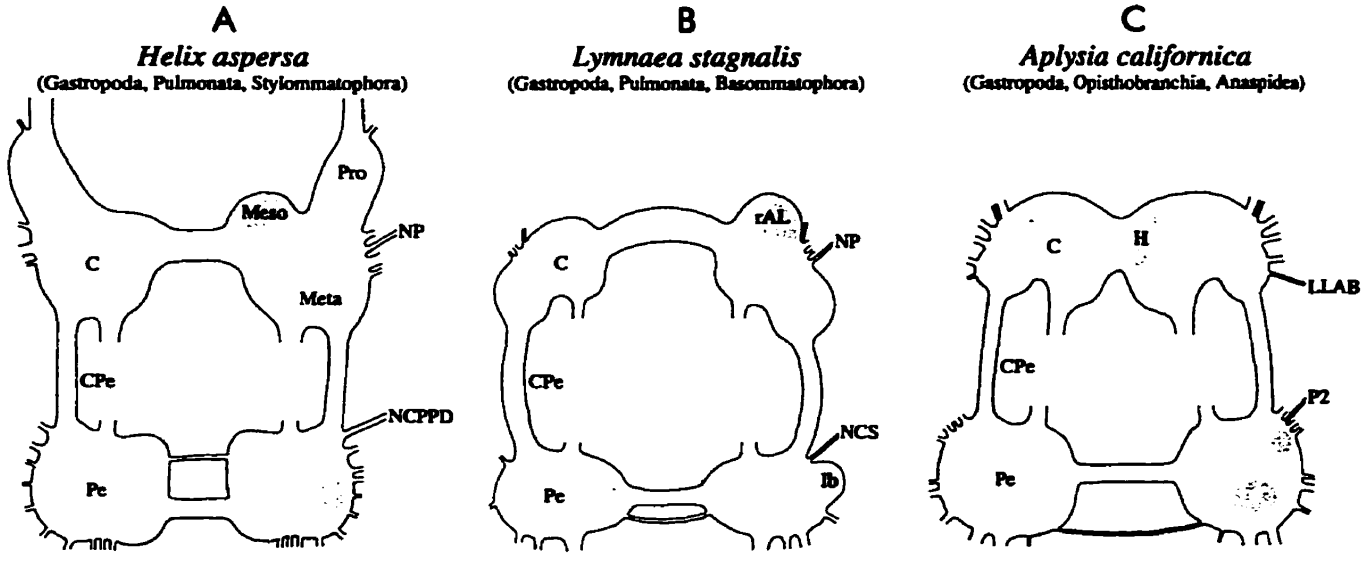


Figure 7. Regions of the CNS controlling mating behaviour in three species of gastropod molluscs. Dorsal views are shown. The shading indicates regions with conserved function, as explained in the Discussion. Nerves are labelled if they are mentioned in the Discussion. Abbreviations: C, cerebral ganglion; LLAB, lower labial nerve; CPe, cerebro-pedal connective; Meso, mesocerebrum, NCPPD, *nervus cutaneus pedalis primus dexter*; NCS, *nervus cervicalis superior*; NP, *nervus penis*; P2, anterior tegumentary nerve; Pe, pedal ganglion; Post, postcerebrum; Pro, procerebrum.

	electrical only N=44	electrical + tactile N=10	tactile only N=13
genital eversion	52.3 %	20 %	0 %
genital eversion and penial eversion	6.8 %	60 %	0 %
genital eversion, dart shooting and penial eversion	0 %	20 %	0 %
no effect	40.9 %	0 %	100 %

Table 1. Evoked eversion of the genital pore by electrical stimulation of the right mesocerebrum with and without tactile stimulation. Electrical stimulation caused eversion of the genital pore similar to that seen during courtship. In two cases this was followed by penial eversion. When electrical stimulation was combined with tactile stimulation on the genital pore, penial eversion was reliably evoked and, in two cases, dart shooting also occurred. Tactile stimulation alone evoked neither genital eversion nor penial eversion. Percentage values indicate successful trials relative to total trials.

	APGW N=24	FMRF N=9	APGW+FMRF N=15	SALINE N=5
genital eversion	91.7 %	0 %	33.3%	0 %
no effect	8.3 %	100 %	66.7%	100 %

Table 2. Eversion of the genital pore evoked by neuropeptide injections. Injections of APGWamide caused genital eversions with mean durations of 8.59 ± 2.97 min. FMRFamide had no obvious effect, but injections combining APGWamide and FMRFamide evoked fewer eversions than APGWamide alone, with mean durations of 9.12 ± 1.79 min. Injections of similar volumes of saline were used as a control. Percentage values indicate successful trials relative to total trials.

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Chapter 7

GENERAL DISCUSSION

The goal of the research presented in this thesis is to come to an understanding of dart shooting in the garden snail *Helix aspersa*. Previous researchers have proposed a variety of hypotheses for why snails shoot so-called love darts. In Chapters 2, 3, and 4, I test three hypotheses that seem to give an adaptive explanation for the phenomenon of dart shooting. I provide evidence that leads me to conclude that the love dart of *Helix aspersa* serves as a vehicle to introduce a bioactive substance into the mating partner, most likely to influence sperm storage. Specifically, I demonstrate in Chapter 2 that the calcareous dart is not a nuptial gift of calcium to assist the mating partner in egg production. Rather than the calcium of the love dart, it is the mucus carried on the dart that has significant effects on the female reproductive tract of the recipient (Chapter 3). The nature of these effects can lead to an increase in the number of sperm that reach the sperm storage site and avoid digestion. These results can be interpreted as a form of either mate manipulation or mate choice. Therefore, in Chapter 4, I apply the evolutionary game theory to dart shooting to tease apart these two alternative hypotheses. Based on the developed game of darts, and empirical findings, I argue that the observed effects of the mucus represent a form of mate manipulation. Additionally, in Chapter 5, I propose to use the term "allohormone" for bioactive substances, as the one transferred in the mucus of the dart, that directly affect the recipient's physiology.

Having come to a better understanding of why darts are shot, I then turned to the neurobiological aspects of dart shooting (Chapter 6). To discover how dart shooting and the other mating behaviours are controlled and expressed by the central nervous system, I adapted the *in vivo* recording and stimulation technique for use in land snails. This method allowed me to test the involvement of the right mesocerebrum in mating behaviour of intact freely behaving animals. In agreement with previous *in vitro* experiments, my *in vivo* results indicate that dart shooting and other mating behaviours - like genital eversion and penial eversion - are controlled by the right mesocerebrum.

In the following, I shall discuss recent experiments done by other researchers in the context of the mate manipulation hypothesis for dart shooting and show that these findings are consistent with my hypothesis. While I believe that the dart is not involved in mate choice, it is likely that in snails, as in many animals, potential mating partners are assessed before mating. Therefore, it is appropriate to discuss here the mechanisms that

are possibly involved in mate choice in snails. This will be followed by a comparison of dart shooting with examples, from other species, of mate manipulation through hypodermic injection of bioactive substances. As for the neuroethological work, the role of APGWamide has been discussed extensively in Chapter 6. Because the role of FMRFamide in the mating behaviour of snails still remains to be determined, I shall speculate on a possible function for this neuropeptide. Finally, I shall make some suggestions for future research on questions that are raised by work presented in this thesis.

Behavioural aspects of dart shooting and mating

Mate manipulation in snails

Sperm competition, which includes manipulation of the mating partner, has received a lot of attention in sexual selection research since Parker published his "seminal" review in 1970. Interestingly, in hermaphroditic species, probably because of the union of the sexes, very little research has been done on this topic (reviewed by Michiels, 1998). Hence, dart shooting, which is confined to hermaphroditic snails, is a very promising subject for further studies of sexual selection in hermaphrodites.

Snails mate promiscuously and store sperm for prolonged periods of time before fertilising their eggs, thus allowing sperm competition processes to evolve that can increase the fertilisation chances of a sperm donor. My results indicate that the dart evolved as a result of such competition. In Chapter 2, I overturn the previous belief that the dart is a gift of calcium. The dart, which is rarely internalised by the recipient, contains an insignificant amount of calcium relative to the requirements for the production of an egg clutch. Therefore, I investigate in Chapter 3 whether the mucus of the dart contains a bioactive substance, an idea that was first put forward in 1925 by Dorello who referred to the substance as *digitana*. I demonstrate that the mucus causes important conformational changes in the female reproductive tract of the recipient. First, my results show that the mucus causes peristaltic contractions in the organ that receives the spermatophore. This peristalsis probably facilitates spermatophore uptake by the partner because snails in which the dart did not penetrate the skin were more likely to

have the tail of the spermatophore left protruding from the genital pore (Chapter 3). Second, the mucus causes the copulatory canal to reconfigure so as to close off the entrance to the gametolytic bursa copulatrix, which should allow more sperm to escape digestion and reach the site of sperm storage. This reconfiguration may change the appearance of the genital eversion because the opening of the copulatory canal can be seen in the higher stages of the eversion. Possibly, this explains the increase in eversion that can be observed when the mucus is injected into the haemolymph of sexually active snails (Chung, 1986; Adamo & Chase, 1990). The fact that dart shooting is an optional component of mating behaviour - snails do not always shoot their darts - is consistent with the mate manipulation hypothesis and not with the mate choice hypothesis (Chapter 4).

Given the effects of the dart's mucus on the female reproductive tract it is expected that more sperm will escape digestion and reach the spermatheca. The spermatheca of *Helix aspersa* consists of 6 to 12 tubules in which sperm can be stored (Rogers & Chase, unpublished observations). Results recently obtained by Rogers and Chase (unpublished observations) reveal a significant effect of dart shooting success on the number of sperm stored in the spermatheca (N=26). Additional proof that more sperm reach the spermatheca when a dart is received comes from paternity tests performed by Landolfa *et al.* (unpublished data). If more sperm reach the storage site when a dart is shot, differences in dart shooting success should be reflected in paternity. In other words, snails that hit their partners should sire more offspring than those that miss their partners when they shoot their dart. This hypothesis was tested by mating snails sequentially with two different "fathers" and determining paternity of the offspring using an allozyme-based test (Landolfa *et al.*, unpublished data). The results showed that snails that shot their darts deep into the "mother" snail sired more offspring than less successful shooters. Although the sample size was small (N=22) and the results were just significant, their data provide the first direct evidence for the suggested function of the dart. One problem encountered in this paternity study was that the snails were collected in the field and had an unknown mating history (i.e. non-virgins). Therefore, sperm stored from previous matings in the field may have distorted the results. Nonetheless, the findings of Landolfa *et al.* encourage further research on this topic.

Landolfa *et al.* assumed that the depth and duration of penetration of the dart are good measures of the amount of mucus that is transferred into the recipient. Normally, the dart carries approximately 2.2 mg of mucus and only half of that amount is needed to cause the effect in the *in vitro* preparation (Figure 2 of Chapter 3). A further improvement of the paternity study mentioned above can be made if one is able to control the amount of mucus introduced into the recipient's body. One could repeat the experiment using snails whose digitiform glands have been surgically removed and which, therefore, shoot "dry" darts (Adamo & Chase, 1990). By experimentally injecting a known amount of the mucus extract, or the isolated active component (see below), the introduced amount can be optimally controlled. "Mothers" can be injected - right after copulating with the first or second "father" - and the effect of the bioactive substance on paternity can be determined with either the allozyme-based test used by Landolfa *et al.* (unpublished data) or DNA-fingerprinting.

At the moment we (M-È Fortier, J.M. Koene, G.T. Nagle, S.A. Painter & R. Chase) are in the process of identifying the biologically active substance(s) in the mucus using the bio-assay from Chapter 3. Once the active substance is identified, the paternity experiment mentioned above can be used to verify if this substance indeed affects the survival of the sperm. As in the experiment of Landolfa *et al.*, "fathers" that are paired with the injection should sire more offspring than control "fathers". Alternatively, one can compare the total numbers of sperm that reach the spermatheca in injected snails and control snails (using the methods of Rogers & Chase, unpublished), the prediction being that the former number would be higher. The identification of the substance in the mucus might also resolve whether the dart is used for mate manipulation or mate choice. For the former a peptide resembling one already used in the snail's endocrinology is expected, while for the latter an arbitrary peptide is expected (see Chapter 4; Adamo & Chase, 1996). The finding that the extract of the mucus-producing pedal gland evokes the same effects on the female reproductive tract as the extract of the digitiform glands (Table 1 of Chapter 3) seems consistent with mate manipulation.

Possible mechanisms of mate choice in snails

In the foregoing and in Chapter 4, I have argued that the dart serves as an instrument to manipulate the mating partner. However, in many species individuals assess their mating partners before copulating with them. I do not discount the occurrence of mate choice in snails, but, given the available data, I believe that it is unlikely that the dart is used to evaluate mating partners. In this section, I discuss other facts that are inconsistent with the mate choice hypothesis for dart shooting. Further, I suggest several alternatives that may serve as mate choice mechanisms.

If dart shooting were a basis for mate choice, one would expect that a snail (after having shot its dart) would wait for the partner's signal (dart) before attempting copulation. The opposite occurs; dart shooting is immediately followed by penial eversions and attempts to intromit, even if the partner has not yet reached the same stage of arousal (Chung, 1987; Adamo & Chase, 1988). A snail probably does not wait for its partner to shoot because it is never certain whether or not a dart has been shot. Darts are often missed (22.4% in Chapter 4) or not shot at all (28.7% in Chapter 4). The only unequivocal indication that the partner has passed its dart shooting stage is the occurrence of intromission attempts. Additionally, dart shooting is an optional component of mating behaviour because non-virgin snails do not always shoot in two consecutive matings (Table 4 of Chapter 4).

Furthermore, if snails were selecting their mating partners based on dart shooting, they should prefer mating with partners that shoot a dart deep into the skin. Thus, if the dart were a mating signal, a snail would be expected to abandon its partner to look for a "better" one when no dart is received. However, when snails separate to look for a different mating partner they do so early in courtship before the dart shooting stage is reached (Chung, 1987; personal observations). Moreover, my results show that mating takes place regardless of the success of dart shooting (Chapter 4). A possible explanation for why mating continues in these cases is that the male function can still benefit from donating sperm to the not-shooting partner because male reproductive success is limited by the number of matings, rather than by the male's resources ("Bateman's principle"; Bateman, 1948). A mathematical model of Greeff and Michiels (1999), however, suggests that in hermaphrodites that digest sperm and mate reciprocally Bateman's

principle may not apply. Instead, the male reproductive success can become limited by the amount of resources invested in sperm and will therefore also benefit from mate choice (Greeff & Michiels, 1999).

If dart shooting is not a basis for mate choice, then how can snails evaluate the reproductive quality of prospective mating partners? There are a number of conceivable mate choice mechanisms that would be more practical than the dart (for example, that could be used more than once every six days). Courtship behaviours often serve to obtain information about the condition of the mating partner. Assessing partners in the early stages of courtship, in this case in the approximately 30 minutes before dart shooting, saves time and energy.

One possible choice mechanism is based on the pheromones that are present in the mucus trails that snails leave behind when they locomote. These trails are used by conspecifics to locate mating partners (Chase *et al.*, 1978; Cook, 1994), but also by carnivorous snails to find their prey (Pearce & Gaertner, 1996). Therefore, producing a mucus trail with a large quantity of pheromone may attract more conspecifics, but at the same time more predators, which makes it a costly signal (a requirement for "Zahivian" mate choice; Zahavi, 1975). Hence, snails that produce a large pheromone signal without being predated show their survival ability and should be preferred as mates.

Another mechanism for mate choice may be based on the response of the partner to biting during courtship. In the introductory phase of courtship animals bite each other on the skin around the genital pore (Chung, 1987; Adamo & Chase, 1988). This behaviour continues to elude explanation. In Chapter 4, I suggest that biting could be used to test the sexual interest of the mating partner, as was proposed by Giusti and Lepri (1980; also Giusti & Adreini, 1988). Withdrawal responses are largely suppressed during mating behaviour (Balaban & Chase, 1990), which can be costly because withdrawal into the shell is the snail's main defence against large predators like birds and rodents. If suppression of this withdrawal makes the animal more vulnerable to predation, the degree of suppression shows how much risk the animal is willing to take to mate, thereby revealing its reproductive quality. A simple experiment that might verify the hypothesis that biting is used to test the sexual interest of the partner would involve carefully recording the biting behaviour during courtship. The first prediction is that most of the

biting occurs early in courtship and that the withdrawal responses decrease when courtship advances. The second prediction is that after dart shooting the biting behaviour ceases. Biting might even have a dual function: It could function to test the sexual motivation of the partner and to help the animal determine the best time for dart shooting. The later function suggests that when the partner's withdrawal reflexes are nearly completely suppressed the snail can shoot a dart and be relatively sure of piercing the skin before the partner withdraws. Alternatively, if biting serves simply to excite the mating partner or to demonstrate the biter's quality, it should be observed after dart shooting until the start of copulation.

A third possibility is that a complicated choice mechanism is not needed. For instance, if an animal initiates courtship only when enough sperm or seminal fluid is present for successful copulation, the expression of sexual activity in itself can become a signal of quality. *Helix aspersa* express sexual activity when its autosperm store (the seminal vesicle, also known as the hermaphroditic duct) is filled with sperm (Koene & Chase, 1996). One requirement for the sexual activity to be a signal of quality is that old and abnormal sperm are continuously replaced in the autosperm store to conserve the quality of the sperm, which has been shown to occur in *Helix pomatia* (Lind, 1973). A part of the *nervus intestinalis* branches over the organ (Rogers & Chase, unpublished data). This nerve seems to provide stretch information, when the organ is artificially inflated in a semi-intact preparation (Koene & Chase, 1996), to the central nervous system (possibly the mesocerebrum: Koene & Chase, unpublished data). This would resemble the situation in *Lymnaea stagnalis* in which stretch receptors in the prostate gland mediate information to the central nervous system about the amount of available seminal fluid, via the *nervus penis* (De Boer *et al.*, 1997a). This information regulates the expression of male mating behaviour; the animal will only engage in male copulatory behaviour if enough seminal fluid is present for a successful copulation (De Boer *et al.*, 1997a). Similarly, in *Helix* the amount of stored sperm may determine whether the animal will engage in courtship behaviour. Comparison of the two different modes of sperm transfer shows that, respectively, the seminal fluid and the spermatophore serve as vehicles to transfer and protect the spermatozoa. Sperm in seminal fluid, however, has direct access to the female tract while sperm in the spermatophore does not. Therefore,

the limiting resource for successful copulation is seminal fluid in *Lymnaea stagnalis* but sperm numbers in *Helix aspersa*.

A last choice mechanism involves measuring the size or quality of the donated sperm or spermatophore. Stretch receptors in the female reproductive tract can provide information about the size of the spermatophore. A nice example is found in the bursa copulatrix of the cabbage white butterfly *Pieris rapea crucivora*, where the spermatophore is received (Obara *et al.*, 1975; Sugawara, 1979). Stretch receptors in this butterfly's bursa copulatrix provide information to the central nervous system about the size of the spermatophore. As long as the organ is stretched the female will be sexually unreceptive (Sugawara, 1979), which was also suggested to occur in the checkerspot butterfly *Euphydryas editha* (Labine, 1964). It is possible that the sperm receiving organs of the snail possess stretch receptors that provide information about the size of the received spermatophore. At least the bursa copulatrix is innervated by a branch of the *nervus intestinalis* and afferent activity can be recorded in this nerve (personal observations). However, nothing is known about the innervation of the bursa tract diverticulum, which is the organ where the spermatophore is initially received.

There is a problem that is often encountered with such findings: Should the observed phenomenon be interpreted as mate choice or mate manipulation (Birkhead, 1998)? In order to answer this question, experiments have to be carefully designed to tease the alternative hypotheses apart, as I did in Chapter 4. Unfortunately, this is not always done, as illustrated by the following example.

In the grasshopper *Gomphocerus rufus* stretch reception was held responsible for the unreceptivity of the female after mating (Loher & Huber, 1966), but this has recently been disproved. Instead, chemoreceptors in the female spermatheca seem specifically sensitive to a male accessory gland component transferred along with the spermatophore (called white substance 1 or WS1, Hartmann & Loher, 1999). When present in the spermatheca (but not in the blood) WS1 can suppress remating of the female for up to 14 days by evoking "secondary defence" against courting males. The chemoreceptors convey their information to the central nervous system. Hartmann and Loher (1999) argue that the observed inhibition of mating represents mate manipulation because it is the male's goal to prevent the female from remating with other males, so that his sperm can

fertilise as many eggs as possible. Additionally, they argue that the female regains some control over the duration of unreceptivity by digesting the male substance.

Contrary to Hartmann and Loher's view, one could argue that the suppression of remating is an example of mate choice. The existence of chemoreceptors detecting the male substance could suggest that males are evaluated on the quality or quantity of WS1. Then, this evaluation determines the duration of the inhibition of the female's receptivity and, thereby, the number of egg clutches that are fertilised exclusively with that male's sperm. Hartmann and Loher (1999) suggest that the quantity of the accessory gland substance may vary with the number of times that the male has mated beforehand or with the age of the male, which are both conditions that may affect the quantity or quality of the transferred sperm. The fact that the amount of WS1 is important, but that males are limited in producing it (Hartmann & Loher, 1999), suggest that it may be a costly substance. The male is best off transferring a large amount of WS1 but can only do this when enough resources are available; therefore the amount of WS1 will reflect his overall quality (handicap principle; Zahavi, 1975).

Both of the hypotheses for the function of WS1 are valid explanations, thus clearly illustrating the problem: Is the observed phenomenon mate manipulation or mate choice (Birkhead, 1998)? In Chapter 4, I specifically addressed this question for dart shooting, and I argued that the dart is most likely used as an instrument to manipulate the mating partner.

Hypodermic injection mechanisms similar to dart shooting

In many internally fertilising species, an individual has the opportunity to transfer chemicals during courtship and copulation to influence the behaviour or physiology of the partner. The biological effects - which range from increasing egg laying, to inhibiting remating, to increasing sperm transport or storage - are important for sexual selection (Eberhard & Cordero, 1995). Nonetheless, no clear defining term exists for such bioactive chemicals. In Chapter 5, I proposed to use the term allohormone for this important class of biologically active products. There are many reviews dealing with bioactive substances that influence the mating partner (e.g. Gillott, 1988; Eberhard, 1996). I shall focus the following on a few examples that are comparable to dart shooting, i.e. hypodermic

injection of an allohormone into the mating partner during courtship, to illustrate the generality of the phenomenon.

In Chapter 5, I described the example of hypodermic introduction of an allohormone in some salamander species (*Desmognathus* spp.), using specialised pre-maxillary teeth associated with a gland (Arnold & Houck, 1982). This allohormone has been shown to increase the probability that the female accepts the male's spermatophore (Houck & Reagan, 1990; Houck, 1998). A similar explanation has been proposed for the stinging of the female by the male *Euscorpius* during this scorpion's courtship (Weygoldt, 1977).

Another example of injection of an allohormone is found in the simultaneously hermaphroditic earthworm *Lumbricus terrestris*. This species bears setae on its skin. Setae are small hair-like chitinous structures that primarily aid locomotion by securing hold on the substrate (Stephenson, 1930). However, the setae that are located on the segments that bear the reproductive organs are larger and modified. Each copulatory setum - a chitinous, needle-like structure with a sharp tip - is associated with a protractor muscle, and a setal gland is present at the base (Stephenson, 1921, 1930). Both Feldkamp (1924) and Grove (1925) reported several instances in which these setae had pierced the skin of the partner during copulation and had injected a substance, presumably from the setal gland, into the skin of the mating partner. Feldkamp (1924) suggested that the introduced secretion diffuses into the blood to cause sexual excitement in the partner. Interestingly, Feldkamp (1924) even compared the copulatory setae of the earthworm with the love dart of *Helix aspersa*.

Neurobiological aspects of dart shooting and mating

The experiments presented in Chapter 6 report the first successful *in vivo* multi-unit recordings in terrestrial snails. Previously, *in vivo* recordings were done in the terrestrial slug *Limax maximus* by Gelperin *et al.* (1996), who recorded fluctuations in the field potential of the procerebrum (i.e. olfactory lobe) rather than the activity of individual neuronal units. In the *in vivo* recording during a snail's mating behaviour, I was able to separate out unitary neuronal components from the multi-unit signal and correlate the activity of these individual units with different components of the mating behaviour. Most of the results, including those relating to APGWamide, have already been discussed in Chapter 6. Because the role of FMRFamide in the mating behaviour of snails in still unknown, I shall here review data with respect to this neuropeptide and propose a possible function for it.

A possible function for FMRFamide in mating behaviour

In the molluscan taxonomy, the Opisthobranchia branched off from the Pulmonata approximately 350 million years ago, while the Pulmonata divided further into the Basommatophora and Stylommatophora orders approximately 115 million year ago. Nonetheless, all snails and slugs that have been studied control mating behaviour with the same anteromedial region of the cerebral ganglia (see Chapter 6). This evolutionarily conserved brain region uses APGWamide to mediate eversion of the genitalia, most likely by relaxing the penial musculature (e.g. De Boer *et al.*, 1997b). Because the Stylommatophora are the only dart shooting molluscs and seem to be the only class to contain FMRFamide in this region of the brain, Li and Chase (1995) proposed that dart shooting evolved accompanied by a neural system using FMRFamide to control it. Although the mesocerebrum does control dart shooting, additional to the other mating behaviours, the data in Chapter 6 suggest that FMRFamide is not involved in controlling it. Then, what is the function of this neuropeptide in the mating behaviour of gastropods?

The presence of FMRFamide in the mesocerebrum of *Helix aspersa* has been subject to controversy. Some studies reported its presence in the mesocerebral area (Elekes & Nässel, 1990, Marchand *et al.*, 1991; Li & Chase, 1995) while others were

unable to detect it (Cottrell *et al.*, 1992). It is interesting to note that FMRFamide had also been found in the Ce1 region of the slug *Limax maximus* (Cooke & Gelperin, 1988), a region of the brain that is a homologue of the mesocerebrum. This finding contradicts the hypothesis proposed by Li and Chase because *Limax maximus* does not possess a dart. Moreover, FMRFamide causes contractions in the penial musculature of *Limax maximus* (Krajniak *et al.*, 1989), as it does in the penis of *Helix aspersa* (Lehman & Greenberg, 1987) and the penis retractor muscle of *Lymnaea stagnalis* (Van Golen *et al.*, 1995). Schaefer *et al.* (1985) reported that some neurones in the right pedal ganglion contain FMRFamide in *Aplysia californica*, and those could be the motoneurones for the penis retractor muscle (Rock *et al.*, 1977; see Chapter 6).

Because relaxation of the penial muscles causes eversion (e.g. De Boer *et al.*, 1997b), the contractions caused by FMRFamide probably keep the penial complex inside the animal. FMRFamide was first identified as a cardio-active peptide (Price & Greenberg, 1977), and has subsequently been found to cause contractions in many molluscan muscles (e.g. Price & Greenberg, 1977; Painter, 1982; Lehman & Greenberg, 1987; Cawthorpe & Lukowiak, 1990; Prescott *et al.*, 1997). The so-called "catch" contraction properties of FMRFamide in muscles of some bivalve species is one example and might be relevant here. In both the southern marsh mussel *Geukensia demissa granosissima* and the blue mussel *Mylitus edulis* the neuropeptide causes a catch contraction of the anterior byssus retractor muscle, which attaches the animal to a fixed substrate, for extended periods of time (Painter, 1982). In *Lymnaea stagnalis* FMRFamide also causes a sustained contraction of the penial retractor muscle that lasts as long as the neuropeptide (3×10^{-7} M) is present in the bath holding the muscle; the muscle slowly relaxes when the neuropeptide is washed out (Van Golen *et al.*, 1995). This response is quite similar to the catch muscle response to FMRFamide (3×10^{-6} M) in the above-mentioned bivalves, although the muscle relaxes much slower in the bivalve muscles after removal of the neuropeptide (Painter, 1982). It is tempting to speculate that FMRFamide causes an extended contraction in the penis retractor muscle of *Helix aspersa*, thus holding the penis inside. The penis retractor muscle would be continuously contracted and only relaxed when the penis is everted. When the animal starts mating behaviour, the release of FMRFamide by the mesocerebral neurones would decrease (e.g. unit 11,

Chapter 6), slowly releasing the catch contraction on the penis retractor muscle. Instead, the APGWamide neurones become active, releasing their neuropeptide and causing eversion of the genitalia.

This scenario is consistent with the results of injections of these peptides in *Helix aspersa* (Table 2 of Chapter 6). When a mixture of APGWamide and FMRFamide was injected into the haemolymph, significantly fewer genital eversions were observed than when APGWamide alone was injected. Hence, I hypothesise that the APGWamide-containing neurones are responsible for genital eversion, possibly by a mechanism that leads to relaxation of the penis retractor muscle; while the neurones that contain FMRFamide are responsible for holding the genitalia inside, possibly through a extended contraction of the penis retractor muscle. FMRFamide might have a similar effect on the dart sac to prevent eversion. Of course, the question remains as to what neuropeptide, if any, controls the expression of dart shooting.

Sensory information is necessary for expression of the complete behaviour

The results of the *in vivo* recordings showed that the mesocerebrum also receives sensory information from the skin. Penial eversion, and sometimes dart shooting, could be evoked only when tactile stimulation was applied in addition to the electrical stimulation of the right mesocerebral neurones (Table 1 of Chapter 6). This finding indicated that the tactile stimulus from the partner is essential for the continuation of the behaviour, as was already suggested by Lind's behavioural observations (1976). The necessity of this tactile information can be compared to the neuronal feedback from the reproductive tract during egg laying in *Lymnaea stagnalis* and *Aplysia fasciata* (see Chapter 1). In both of these species the sensory information of the eggs passing through the reproductive tract is necessary for the expression of complete egg laying behaviour (Ferguson *et al.*, 1993; Ter Maat & Ferguson, 1996).

More *in vivo* recordings in *Helix aspersa*?

Several problems were encountered with the *in vivo* recordings in *Helix aspersa*. Because *Helix aspersa* is a terrestrial species, recordings of extracellular signals in these animals suffer from much more noise than recordings from aquatic species. In such recordings most of the noise can be effectively shielded by grounding the water in the aquarium. To cope with this problem in *Helix aspersa* a second fine wire with a free

ending was implanted to function as the reference. Although the presence of this reference wire eliminated much of the noise, the signal-to-noise ratio was not optimal. A more severe problem was experienced with the behaviour of the animals after surgery. Even though the snails were selected for high sexual activity immediately before the operation, sexual motivation severely decreased by the time the animals had recovered from the surgery, and it remained low. This decrease in sexual motivation was the main reason why only two recordings were obtained during spontaneous mating behaviours. Similar effects of the surgery on sexual motivation were encountered in experiments with *Lymnaea stagnalis* (N=2, De Boer *et al.*, 1997b). The operated animals of both species otherwise behaved normally, which indicates that the surgery mainly interfered with the motivational state of the animal. Therefore, future *in vivo* studies in *Helix aspersa* and other molluscs can certainly be done using the methods that I developed. However, I expect that the highest success rate will be obtained with the study of behaviours that are less dependent on motivational control than mating.

Suggestions for future research

As suggested in Chapter 4 and earlier in this chapter, the biting observed during courtship might be an interesting component of mating behaviour to investigate in the context of mate choice. By looking at the exact occurrence and timing of the biting and the accompanying withdrawal of the partner, it can be determined whether biting is used to test the partner's sexual interest. Two topics for further investigation in the research on the mucus of the love dart, also suggested earlier in this chapter, are to biochemically identify the allohormone and to repeat the paternity tests using this identified substance. Also, more thought should be given to the evolution of the love dart in the Stylommatophora. At the end of Chapter 4, I suggest that comparison of the reproductive morphology of different dart shooting species might reveal a co-evolution of the dart and the female reproductive system, possibly representing an arms race. Finally, neurobiological experiments should be done on the H-cluster (the proposed homologue of the mesocerebrum) of *Aplysia californica* to confirm the conclusions of Chapter 6. The H-cluster neurones' projections to the penis, their motor function on the penis, and the involvement of APGWamide need to be demonstrated.

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