DART SHOOTING AND POSTCOPULATORY SEXUAL SELECTION IN THE GARDEN SNAIL *HELIX ASPERSA*

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ABSTRACT

During the final stage of courtship, the garden snail *Helix aspersa* attempts to stab its mating partner with a mucus-coated calcareous "love dart." I present evidence supporting two predictions of the most promising hypothesis for the adaptive significance of this behavior: that the dart serves to increase the reproductive success of the shooter by increasing the numerical representation of its sperm in the recipient's storage organ (the sperm loading hypothesis). First, I demonstrate that once-mated snails store more of the sperm transferred by successful shooters than by unsuccessful shooters. Second, I demonstrate that this biased storage results in higher paternity scores for successful shooters relative to unsuccessful shooters in the clutches of multiply mated recipients. Moreover, I present evidence that body size and mating order influence the outcome of sperm competition in snails. Finally, I propose a novel mechanism to explain the observed pattern of sperm utilization in *H. aspersa* based on the motility of stored allosperm.

RÉSUMÉ

Lors de l'étape finale de la cour, l'escargot de jardin *Helix aspersa* tente de poignarder son partenaire avec une "fléchette d'amour," un dard calcaire enduit de mucus. Je presente des évidences supportant deux prédictions qui écoulent de l'hypothèse la plus prometteuse expliquant la signification adaptative de ce comportement: soit que le dard sert à augmenter le succès reproducteur du tireur en augmentant la quantité de spermatozoïdes accumulée dans l'organe d'emmagasinage du récipiendaire (l'hypothèse du chargement de sperme). D'abord, je démontre que les escargots inseminés une seule fois emmagasinent un plus grand nombre de spermatozoïdes lorsque le tir a été réussi que lorsqui'il a échoué. Deuxièmement, je démontre que ce phénomène engendre un taux de paternité plus élevé chez les escargot ayant réussi leur tir en comparaison de ceux qui ont échoués. De plus, je présente des évidences que la taille corporelle et l'ordre d' accouplement influencent les résultats de la compétition entre les spermatozoïdes. En dernier lieu, je propose un nouveau mécanisme pour expliquer l'utilisation des spermatozoïdes par *H. aspersa*.

FORMAT AND CREDIT WEIGHT

This dissertation consists 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 "Guidelines for Thesis Preparation" by the Faculty of Graduate Studies and Research):

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary;

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".

This thesis carries a credit weight of 39 credits, from a total of 45 credits required for the Master's degree. Graduate credits are a measure of the time assigned to a given task in the graduate program. They are based on the consideration that a term of full-time graduate work is equivalent to 12 to 16 credits, depending on the intensity of the program.

MANUSCRIPTS AND AUTHORSHIP

The research presented in this thesis was conducted exclusively by David W. Rogers under the supervision of Dr. Ronald Chase. All chapters included in this thesis were written by David W. Rogers. Ronald Chase provided valuable insight and editorial help in the writing of all chapters. The comments of Dr. M.A. Landolfa, Dr. J.M. Koene, and two anonymous reviewers helped to improve Chapter 2. However, any errors included in this document are solely the responsibility of the author.

Chapters 1 and 4 were prepared exclusively for this thesis.

Chapter 2 has been previously published and is reproduced here with copyright permission from Springer-Verlag. The original publication is:

Rogers DW, Chase R (2001) Dart receipt promotes sperm storage in the garden snail *Helix aspersa*. *Behavioral Ecology and Sociobiology* 50(2):122-127

Chapter 3, co-authored by Ronald Chase, has been submitted for publication in *Behavioral Ecology and Sociobiology*.

Ronald Chase contributed to the inception and design of experiments and the interpretation of the results presented in Chapters 2 and 3.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. I explain the adaptive significance of dart shooting in *Helix aspersa*. In support of this explanation, I demonstrate that:

(1.1) Snails penetrated by their partners' love darts store significantly more sperm than do snails that are missed (Chapter 2).

(1.2) Dart shooting increases the proportion of offspring fathered by a successful shooter, relative to an unsuccessful shooter, in a competitively fertilized clutch (Chapter 3).

2. I demonstrate the importance of body size in determining the outcome of sperm competition in *H. aspersa*. I present evidence that:

(2.1) The absolute number of sperm stored decreases with increasing shell volume of the recipient (Chapter 2).

(2.2) The number of sperm transferred is dependent on donor body size (Chapter 2). Additionally, I present a novel protocol for determining the number of sperm contained in a spermatophore (Chapter 2).

(2.3) The effect of dart receipt is stronger in small recipients than in large recipients (Chapters 2 and 3).

3. I describe factors other than dart receipt and body size that influence sperm utilization in *H. aspersa*.

(3.1) I present evidence of first donor sperm precedence (Chapter 3)

(3.2) I present a novel mechanism to explain the observed pattern of sperm utilization based on the motility of stored allosperm (Chapter 3). No previous discussion of this mechanism exists in the literature and it has potential implications for the study of sperm competition in many taxa.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the input, encouragement, and critical evaluation of Ronald Chase. On two separate occasions, when I had succumbed to the frustration of repeated failures, he rescued this project from complete ruin. He tolerated my frequent forays into other fields of research, despite the fact that my efforts rarely produced meaningful results, and demonstrated a remarkable ability to discover the flaws in almost all of my ideas. His support and criticism have been invaluable to my growth as a student.

I cannot take credit for most of the ideas presented in this dissertation, as they are the result of hours of discussion with many people. In particular, Ronald Chase, Mike Landolfa, and Joris Koene were, and continue to be, important sources of inspiration. Joris provided the foundation for this research and Mike provided the appropriate context. We have not always agreed on interpretation, but they have never dismissed my opinions.

I thank the other members of my supervisory committee. Derek Roff provided help with statistics and Donald Kramer ensured that I took nothing for granted. I am also indebted to Angus Davison for his instruction in molecular techniques and David Green for the use of his equipment and facilities. The research presented in Chapter 2 would have been impossible without the help of Robert Levine and Stéphanie Ratté. K. Fenelon, L. Zaccardelli, G. M. Ntim, and my parents helped in the collection and care of snails. Additionally, I thank NSERC Canada for financial support.

Finally, I am deeply grateful to Jason Burgess and Ursula Oldenburg for giving me reasons to occasionally leave the lab.

Chapter 1

GENERAL INTRODUCTION

Despite a multitude of descriptions of the sexual behavior of land snails, little is known about the evolutionary significance of any aspect of courtship or copulation in these animals. In particular, no satisfactory explanation has been provided for the adaptive significance of dart shooting in helicid snails. In this thesis, I attempt to understand the ultimate function of dart shooting and identify other morphological and behavioral traits that are subject to "broad-sense" sexual selection (Civetta and Singh 1999).

Courtship and copulation in the garden snail

The garden snail *Helix aspersa* Müller (Gastropoda: Pulmonata: Stylommatophora: Helicidae) is a simultaneously reciprocal hermaphrodite. Mating partners simultaneously exchange sperm-filled spermatophores during copulation. A prolonged and elaborate courtship precedes this exchange. When two sexually receptive snails meet they engage in introductory behavior (Adamo and Chase 1988) consisting of lip-lip contact, lip-genital contact and occasional biting. During this time, the genital atrium of each snail tumesces and begins to evert (Fig. 1). As courtship proceeds, the extent of atrial eversion increases until one snail presses tightly against the body wall of its mating partner, assumes a characteristic posture (pre-dart shooting posture, Adamo and Chase 1988), and attempts to plunge a sharp 9-mm long calcareous spicule through the partner's skin (Fig. 1).

This spicule, colorfully termed the "love dart" (hereafter referred to as the dart), is forcefully expelled through the shooter's genital pore by evagination of the muscular dart sac which secretes and stores the dart prior to use (Hunt 1979). As the dart is shot, it is coated with approximately 2 mg of white mucus secreted from the digitiform glands

(Chung 1986a). This mucus is transferred into the hemocoel of successfully darted recipients (Adamo and Chase 1990). Shot darts, however, sometimes fail to puncture the skin of the intended recipient (~30% miss, D. Rogers, personal observation), in which case they are generally retracted by the shooter (Fig. 1). Retraction is possible because the dart remains attached to the evaginated dart sac via the tubercle until contact with the partner's skin causes it to become detached (i.e. the dart is stabbed rather than shot). However, retracted darts cannot be used in subsequent matings. Instead, they are slowly digested by the shooter and new darts are produced over the next six days (Tompa 1982). Production of the dart is initiated by eversion of the dart sac during courtship. Consequently, virgin snails do not possess darts (Chung 1986b).

Once the first snail has shot, it immediately attempts to initiate copulation by intromitting its penis into its partner's genital pore. These attempts prove futile until the second snail shoots, after which reciprocal intromission is established and copulation begins. During copulation, a proteinaceous spermatophore shell is produced in the epiphallus and flagellum (see Fig. 2 for a diagram of the reproductive anatomy), filled with autosperm from the seminal vesicle, and transferred via the penis into the partner's female reproductive tract (Lind 1973). While spermatophore production occurs rapidly, transfer is not initiated until approximately 4 hours after the start of copulation (Adamo and Chase 1988). The entire duration of copulation is approximately 7 hours (Adamo and Chase 1988).

The spermatophore is received into the partner's bursa tract diverticulum (Fig. 2). At this point the allosperm, quiescent during transfer, become active through an unknown mechanism and swim down the long tail of the spermatophore to the copulatory canal. Most sperm are drawn, through peristalsis in the bursa tract, into the gametolytic bursa

copulatrix where they are digested (Nemeth and Kovacs 1972, Lind 1973). However, a small proportion of the transferred sperm (estimated at 0.1% by Lind 1973) escape digestion and travel up the spermoviduct to the fertilization pouch spermatheca complex (FPSC, Tompa 1984) where they are stored in elongate blind-ended tubules until required for fertilization (Lind 1973). Stored allosperm remain capable of fertilization for up to four years (Duncan 1975).

H. aspersa mates with multiple partners between oviposition events (Moulin 1980, Madec et al. 1998), and sperm from different donors are stored simultaneously in the spermathecal tubules (Murray 1964). While the focus of this section is dart shooting, I will briefly review the benefits of mating with multiple partners among snails and other simultaneous hermaphrodites.

Sex roles in hermaphrodites

It is generally assumed that simultaneous hermaphrodites mate primarily to donate sperm to their mating partners (Charnov 1979). This assumption stems from Bateman's (1948) principle that while female fecundity is generally limited by resource availability, male fecundity is generally limited by the availability of females (or their eggs). The strategic difference can be attributed to anisogamy. Since it is usually metabolically cheaper to produce a single ejaculate than it is to produce a clutch of eggs, males can afford to mate rather indiscriminately while female are expected to be choosy. Indeed, resource allocation to gametes in land snails is highly female biased (>95%; Locher and Baur 2000) indicating that sperm production is relatively inexpensive. Moreover, while *H. aspersa* donates sperm to multiple (range: 1-6) partners each breeding season, it generally lays only one clutch of eggs (Madec et al. 1998).

While the benefit of multiple mating to male reproductive success is obvious, let us examine the reasons snails might copulate multiply in order to receive sperm. Snails do not exhibit paternal care, therefore multiple mating can benefit female reproductive success in two ways: direct fertility benefits and genetic benefits. Long-term sperm storage uncouples copulation and oviposition in land snails. That is, copulation is not required to stimulate egg-laying. Moreover, the sperm stored from a single mating can be used to fertilize multiple clutches (Chen and Baur 1993) suggesting that restoring depleted allosperm reserves is not a primary reason to copulate. Greeff and Michiels (1999a) hypothesized that the resources acquired through sperm digestion might be used to increase female fecundity. However, since simultaneously reciprocal hermaphrodites always donate sperm during copulation, the inefficiency of energy transfer requires that they will always suffer a net loss. Indeed, snails likely recuperate only about 10% of the energy invested in an ejaculate through sperm digestion. Since the energy demands of spermatophore production are small (compared to egg production), the increase in female fecundity gained through sperm digestion would be negligible even if snails were able to only receive sperm during mating. Consequently, direct fertility benefits are not an important reason for snails to mate multiply.

Snails might increase their reproductive success through receiving sperm from multiple donors by improving the genetic quality of their offspring. Preferentially using the sperm of high viability or 'attractive' donors can increase offspring fitness (see Chapter 4). Alternatively, producing genetically diverse offspring might increase female reproductive success through genetic bet-hedging (Yasui 1998). A snail's environment is highly variable across both space and time. Small size and limited dispersal ability renders most environments spatially coarse-grained even over small distances. Moreover,

snails are sensitive to small temporal fluctuation particularly in temperature and humidity. Given this unpredictable environment, it seems unlikely that snails are able to foresee which "good genes" will be required by the next generation. Consequently, sire-selection might not have a large effect on female reproductive success. Instead, snails might store sperm from a diverse group of donors, independent of donor phenotype, in order to produce at least some offspring adapted to the environment confronted by the next generation.

Despite the potential benefits of multiple mating to female reproductive success, most simultaneous hermaphrodites probably do copulate primarily to donate sperm. This assumption is supported by behavioral analysis of the sequentially reciprocal sea hare *Aplysia*. Susswein et al. (1993) found that in any given mating pair, the animal exhibiting higher sexual drive assumed the male role. Perhaps the best evidence that *H. aspersa* copulates primarily to donate sperm stems from observations of unilateral penis intromission (reciprocal intromission must be simultaneous). Chung (1987) reported that while the snail that achieved intromission adopted the normal copulatory posture, the snail acting only as a recipient pulled away and attempted to bite its partner's penis until it was dislodged. That is, *H. aspersa* is willing to exclusively adopt the male, but not the female, role.

Nevertheless, sex-role reversal can occur in simultaneous hermaphrodites. Greeff and Michiels (1999a) hypothesized that, under certain conditions, high levels of sperm digestion can trigger an arms race. To increase the number of sperm reaching the recipient's storage organ, the male component evolves larger spermatophores. In response, the female component evolves to digest larger numbers of received sperm. Although Greeff and Michiels (1999a) argued that increased sperm digestion benefits

females through resource accrual, I suspect that females digest sperm primarily to avoid high storage costs (see Chapter 4). Whatever the reason for sperm digestion, this arms race might result in male-biased sex allocation (sex-role reversal). That is, spermatophore production can become costly. Under these circumstances, we would expect simultaneous hermaphrodites to discriminate between potential partners prior to sperm donation. Although there is evidence for sex-role reversal in certain species (the sea slug *Navanax inermis*, Leonard and Lukowiak 1985; the planarian flatworm *Dugesia polychroa*, Michiels and Bakovsky 2000), these appear to be exceptions.

The adaptive significance of dart shooting

Dart shooting has intrigued scientists, philosophers and naturalists for almost three centuries (reviewed by Kothbauer 1988). Over this period, many hypotheses for the function of the dart have been proposed but few have been investigated. Most early ideas were based on analyses of preserved specimens and seem laughable in the light of current knowledge. Indeed, Ashford (1883, p.76) suggested that when these hypotheses were developed "Imagination frequently acted as Reason's substitute." At the time of Ashford's review there was still controversy over whether or not the dart is sufficiently robust to penetrate the skin of the recipient. During nearly a century and a half of scientific inquiry nobody bothered to observe the actual behavior! Nevertheless, several of the proposed hypotheses are plausible. In this section, I evaluate the most popular ideas and indicate which are most promising.

Reproductive isolation

The molluscs, comprising over 120,000 extant species, represent the second most diverse phylum in the animal kingdom, surpassed only be the insects (Baur 1998). Of these species, 105,000 belong to the gastropod class (Salvini-Plawen 1985). This diversity renders taxonomic differentiation difficult as multiple species often occur sympatrically and exhibit little variation in anatomical characters. Surprisingly, given the conserved nature of most morphological traits in snails, dart structure varies considerably between closely related species. In fact, the dart is often the best way to differentiate between species (e.g. *Cepaea nemoralis* and *Cepaea hortensis*, Tompa 1980). The species specificity of dart structure has inspired the hypothesis that dart receipt allows snails to recognize conspecifics during courtship leading to the avoidance of hybridization and promoting reproductive isolation between species (Diver 1940, Webb 1952).

While species recognition is probably one of the functions of courtship, dart shooting seems ill suited to this purpose. There is no specialized sensory organ responsible for dart receipt. Rather, the location of penetration varies widely between the sole of the foot, the head and the right side of the animal. It is improbable that the recipient is able to determine the exact morphology of the dart based on little more than a puncture wound in the skin. Thus, variation in dart structure is unlikely to promote reproductive isolation. The lack of sensory sophistication applies only to judgment of the shape of the dart. Snails might be able to recognize conspecifics based on the composition of the digitiform mucus that circulates within the hemocoel of darted animals. However, two further pieces of evidence cast doubt on this hypothesis. First, dart shooting occurs at the end of protracted courtship, which can require in excess of 24 hours in *H. aspersa*

(although the mean duration is roughly 1 hour; Adamo and Chase 1988). Thus, by the time dart shooting occurs, snails have already expended considerable time and energy. Species recognition almost surely occurs during the introductory stages of courtship, prior to the investment of substantial resources. Indeed, reports of interspecific pairings between closely related species are extremely rare among land snails (I am aware of only two published accounts: Lang 1908, Webb 1951), suggesting that species recognition occurs long before dart shooting. Second, and perhaps more important, the likelihood of successful copulation is independent of dart receipt (Landolfa in press). If dart shooting served to prevent costly interspecific matings, we would expect snails to refuse to copulate if not struck by their partners' darts.

The available evidence does not support an association between dart shooting and species recognition and the remarkable structural diversity of darts remains to be explained (see Chapter 4). Although the morphology of darts varies considerably, the composition does not. All darts examined to date are composed of the calcium carbonate crystal aragonite (Tompa 1984), a characteristic that has inspired an alternate hypothesis for the adaptive function of dart shooting.

Gift of calcium

Calcium availability limits the rates of reproduction, development and growth in *H. aspersa* (Crowell 1973). Consequently, supplying a mating partner with calcium might augment the number or viability of its offspring thereby increasing the male reproductive success of the calcium donor. Inspired by contemporary work on insects (e.g. Thornhill 1976), Charnov (1979) proposed that the calcareous dart acts as such a gift in snails. However, Koene and Chase (1998a) demonstrated that the dart contains roughly the same

amount of calcium as a single egg (dart = 0.37 mg, egg = 0.41 mg). Since the average clutch size in *H. aspersa* is 59 eggs (Koene and Chase 1998a), the calcium transferred in the dart is unlikely to have important consequences on the female reproductive success of the recipient. Moreover, Koene and Chase (1998a) reported that only 6.3% of shot darts are internalized by the recipient indicating that this small amount of calcium is usually wasted.

Even without the observations of Koene and Chase (1998a), there is reason to doubt Charnov's (1979) hypothesis. In order to benefit the donor, the transfer of a nuptial gift must provide the donor with a level of paternity assurance. That is, increasing the reproductive output of the recipient is adaptive only if the recipient uses sperm from the gift-giver - as opposed to those from a different donor – for fertilization. For instance, in nuptial feeding insects (hanging flies, Thornhill 1976; decorated crickets, Sakaluk 1985) the duration of insemination is highly correlated with the size of the nuptial gift. That is, females store more sperm from males offering larger gifts resulting in higher paternity scores for more 'generous' males. Additionally, males produce larger gifts when the risk of sperm competition is low and the corresponding assurance of paternity is high (Simmons 1995). Paternity assurance is also required when the nuptial gift is transferred in the semen. Female moths (*Utetheisa ornatrix*, Iyengar and Eisner 1999) preferentially mate with males offering large nuptial gifts of a pyrrolizidine alkaloid which is incorporated into eggs rendering them less vulnerable to predation. Most snails are highly promiscuous and possess no obvious mechanism of paternity assurance. In fact, digestion of internalized darts is a very slow process, requiring several months for the received dart to be completely dissolved (D. Rogers, personal observation). Since the interval between copulation and egg laying (mean ± SD = 22 ± 24 days; Koene and Chase 1998a) is

generally considerably shorter than the time required to dissolve the dart, it seems unlikely that the calcium in the dart would primarily benefit its shooter.

I have presented considerable evidence against the hypothesis that the dart serves as a nuptial gift of calcium. However, we cannot cast aside the importance of calcium altogether. Since the calcium in the dart is a limited resource, dart production exacts a cost on the shooter. It remains possible that the dart is a costly and therefore honest signal of the innate quality of the shooter that serves as a basis for mate choice in snails (Charnov 1979).

Honest signal

Few examples of mate choice in hermaphrodites have been documented. If, as argued above, simultaneous hermaphrodites copulate primarily to donate sperm there would be little selective pressure to refuse any partner, assuming that sperm production is relatively inexpensive (Greeff and Michiels 1999b). However, mate choice is expected among sex-role reversed hermaphrodites, where performing the male role exacts a considerable cost. For instance, certain planarian flatworms indicate their fecundity during courtship by adopting a flattened posture (Vreys and Michiels 1997). Worms refuse to mate with partners smaller than themselves, and this pre-copulatory choice results in pronounced size-assortative mating. Since fecundity in highly correlated with body size in this species, this courtship display serves as an honest signal. Based on a questionable presumption, Leonard (1992) suggested that all simultaneous hermaphrodites exhibiting internal fertilization and sperm storage, including helicid snails, are sex-role reversed. Accordingly, she hypothesized that dart shooting serves as an honest signal of a snail's intention to donate sperm. This assurance of sperm transfer,

she claimed, would induce the partner to reciprocate and both snails would benefit by receiving valuable allosperm. Although Adamo and Chase (1996) refuted several predictions based on Leonard's hypothesis, the relationship between dart shooting and sperm transfer has not been investigated (see Chapter 2). It remains possible that variability in ejaculate quality is reflected by dart shooting ability (the phenotype-limited functional fertility hypothesis, Sheldon 1994). However, as noted above, direct fertility benefits are of limited importance to simultaneously reciprocal hermaphrodites.

Although hermaphroditic species with typical sexual roles are likely to donate sperm indiscriminately, information on the innate quality of a mating partner garnered through precopulatory displays might influence utilization of sperm by the recipient. That is, while the potential for precopulatory mate choice is low, the potential for postcopulatory (cryptic) mate choice is not. Snails would benefit, through increased reproductive success, by using the sperm of higher quality donors for fertilization. Whether or not dart shooting success reflects the viability of the sperm donor, either through the aforementioned calcium hypothesis or some other mechanism, remains unknown. Honest signalling, therefore, is a candidate hypothesis for dart function (Landolfa in press). However, as noted above, if the environment faced by subsequent generations is highly unpredictable, viability-based mate choice is unlikely to benefit female reproductive success.

Sexual stimulation

In gonochoristic species, males use courtship to coerce females into copulating. Females, burdened with higher investment in producing and rearing offspring than males, are generally reluctant to mate. Consequently, selection should favor adaptations that

allow males to sexually stimulate their mating partners. Maupertuis (1745) first suggested such a role for the dart, proposing that the mechanical stimulation of dart receipt awoke 'the passion' of these otherwise lethargic creatures. This hypothesis, re-iterated by Ashford (1883), was first tested by Goddard (1962) who found that pinching the skin near the genital pore (a common location of dart receipt) increased the tonus of the penial muscles. Dorello (1925) suggested that the injection of digitiform mucus by the dart, rather than the mechanical stimulation of receipt, was responsible for sexual stimulation of the partner. Indeed, Chung (1986a) found that snails evert their genital atria and/or penes in response to injection of digitiform gland extract. Additionally, Börnchen (1967) noted that such injections increase the frequency and amplitude of heart contractions in the recipient.

Direct studies of the relationship between dart shooting and sexual arousal have largely failed to support the sexual stimulation hypothesis. Lind (1976) observed that snails became less active in courtship when struck by darts. Also, Chung (1987) noted that darted snails exhibited fewer attempts at copulation (penis eversion) than did those not hit by a dart. Indeed, there is no evidence that dart shooting success increases the probability of successful copulation. However, the work of Adamo and Chase (1988) does provide a level of support for this hypothesis. They found that the interval between the first and second shot was shorter if the first shot hit rather than missed the recipient. This decrease in courtship duration was later shown to be dependent on the digitform mucus rather than the mechanical stimulation of dart receipt (Adamo and Chase 1990). While decreasing mating duration might reduce both the risk of predation and energy expenditure, successful shooting reduced the duration of an average mating episode by only 5.5% (Adamo and Chase 1988).

Not only is the sexual stimulation hypothesis not supported by the available evidence, but it is also logically untenable. First, the dart is shot near the end of courtship, long after the mating partner has exhibited sexual motivation. Indeed, it is my impression that a snail will not shoot its dart until it has established that its partner is willing to copulate. Second, while females are expected to be reluctant mates, males are not. If most simultaneous hermaphrodites are expected to copulate primarily to fulfill the male role, snails should be eager to mate and donate sperm. No coercive measures are necessary to promote copulation. However, once sperm have been transferred, their fate is determined by the recipient (acting in the female role). The dart might influence this decision.

Sperm loading

While the studies outlined above do not support the sexual stimulation hypothesis, they do indicate that the dart - or more specifically, the digitiform mucus coating the dart – has a physiological effect on the recipient. Inspired by these early studies, Koene and Chase (1998b) exposed the reproductive tract to a homogenate of the digitiform glands *in vitro*. Upon application of the homogenate, they observed an increase in the rate of peristalsis in the bursa tract diverticulum and conformational changes in the copulatory canal. They speculated that these changes would speed the uptake of the spermatophore by the recipient and close off the entrance to the gametolytic bursa copulatrix (see Fig. 2). These changes are consistent with the hypothesis first proposed by Chung (1987) and later expanded by Adamo and Chase (1996) that dart receipt promotes survival, and eventual storage, of the shooter's sperm in the reproductive tract of the recipient.

Although logically sound and consistent with the available evidence, this hypothesis has not yet been tested (see Chapter 2).

Evidence that substances transferred by males promote the uptake and storage of sperm within the female tract stems from studies on mammals and insects. Prostaglandins in mammalian seminal fluid can stimulate the contraction of the muscular female reproductive tract required for sperm transport (Drobnis and Overstreet 1992). The male assassin bug *Rhodnius prolixus* transfers a serotonin-like seminal factor (indolakyl amine) to the female that causes contraction of the oviduct, transporting the inseminated sperm to the storage organs (Davey 1958). Additionally, the subcutaneous injection of male substances is not restricted to dart shooting snails. Saw-like structures on the paraphallus of the blowfly *Lucilla sericata* pierce the wall of the female bursa and inject accessory gland substances that reduce female re-mating proclivity (Lewis and Pollock 1975). The male plethondontid salamander *Desmognathus fuscus* induces the female to accept his spermatophore through the transfer of mental gland secretions by means of biting her with his gland-associated pre-maxillary teeth (Houck and Reagan 1990). Finally, the simultaneously hermaphroditic earthworm *Lumbricus terrestris* injects accessory gland material through the body wall of its mating partner using specialized copulatory setae (J. M. Koene, unpublished data). All of these adaptations appear to have evolved in response to sperm competition.

Sperm competition is defined as competition within a single female between the sperm of two or more males for the fertilization of ova (Parker 1970). Parker (1970) predicted that the highest levels of sperm competition would occur in systems characterized by: (a) multiple mating by females prior to fertilization, (b) storage of sperm from multiple donors within the female, (c) long term viability of stored sperm,

and (d) efficient utilization of stored sperm during fertilization. Evidence has been presented above indicating that *H. aspersa* meets all of these criteria. Despite the high potential for sperm competition in snails, almost nothing is known about its underlying mechanisms or evolutionary consequences. Only intermating interval has been identified as a factor influencing paternity in clutches fertilized by multiple donors (Baur 1994). The hypothesis that the dart receipt promotes sperm storage suggests a role for the dart in sperm competition. By successfully darting its mating partner, a snail might increase its fertilization success by increasing the numerical representation of its sperm within the storage organ with respect to rival sperm from unsuccessful shooters (sperm loading, Dickinson 1986).

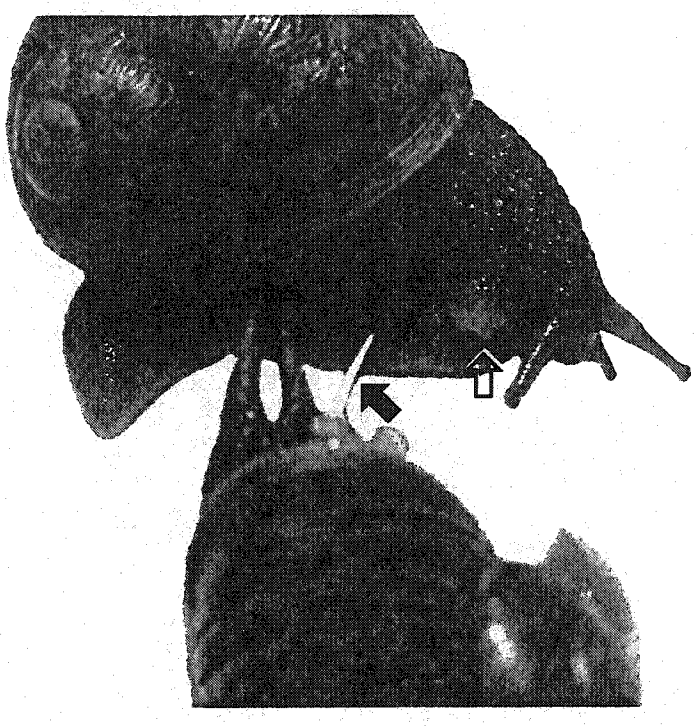
Thesis objectives

The primary objective of this thesis is to test the sperm loading hypothesis for the adaptive significance of dart shooting. Two predictions can be drawn from the sperm loading hypothesis. First, snails store more of the sperm transferred by successful shooters than by unsuccessful shooters. Second, this biased storage results in higher paternity scores for successful shooters, compared to unsuccessful shooters, in competitively fertilized clutches. Tests of these predictions are described in Chapter 2 and Chapter 3, respectively.

The secondary objective is to identify factors influencing the outcome of sperm competition in *H. aspersa*. These are described in both Chapter 2 and 3. Finally, in Chapter 4, I provide a general discussion of the results with an emphasis on issues not addressed in the other chapters.

[Figure on next page]

Fig. 1 Dart shooting in *Helix aspersa*. The lower snail has attempted to stab the upper snail with its dart (solid arrow). The dart, which failed to penetrate the skin of the recipient, will be retracted and either slowly digested or eventually expelled by the shooter. Having recently shot, the lower snail still exhibits the characteristic pre-dart shooting posture (note the forward-pointing tentacles). The everted genital atrium of the upper snail is indicated by the hollow arrow. Reproduced with permission from M.A. Landolfa.



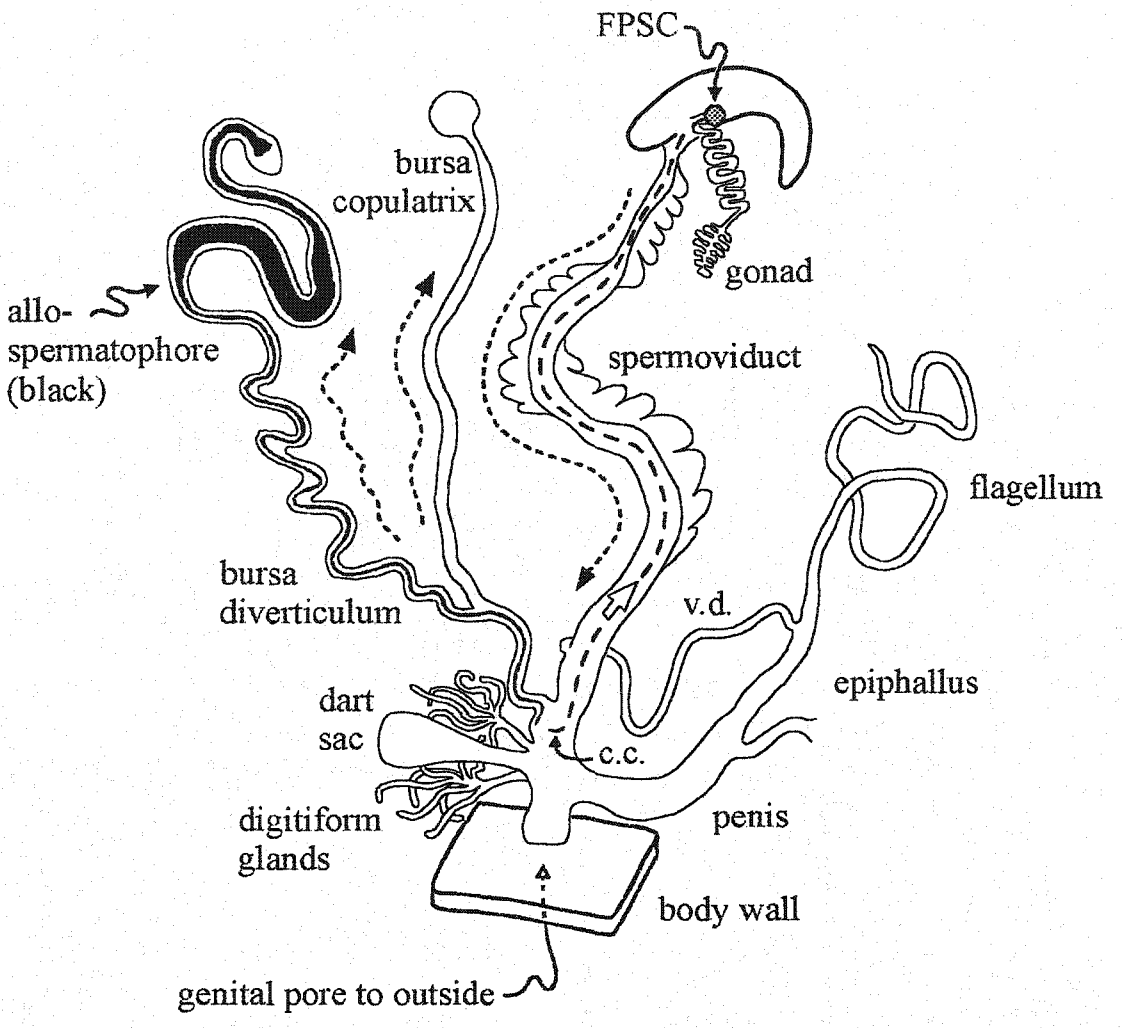


Fig. 2 Semi-schematic representation of the internal reproductive anatomy of *Helix aspersa* immediately after the termination of spermatophore transfer. The received spermatophore, located in the bursa diverticulum, is shown in black. The dotted line (hollow arrowhead) indicates the path traveled by allosperm, from the tail of the spermatophore in the copulatory canal to the sperm storage organ (FPSC), against the direction of peristalsis in the reproductive tract (indicated by the black dotted lines with filled arrowheads). c.c., copulatory canal; v.d., vas deferens. Reproduced with permission from M.A. Landolfa.

Chapter 2

DART RECEIPT PROMOTES SPERM STORAGE IN THE GARDEN SNAIL *HELIX ASPERSA*

In this chapter, I test the first prediction of the sperm loading hypothesis : that snails store more of the sperm transferred by successful shooters than by unsuccessful shooters. Moreover, I attempt to identify other factors influencing both the number of sperm transferred during copulation and the number of sperm stored following spermatophore receipt.

Abstract

During courtship, many helicid snails attempt to pierce the body walls of their mating partners with mucus-coated calcareous darts. The mucus covering the dart induces conformational changes in the female reproductive tract of the recipient, closing off the entrance to the gametolytic bursa copulatrix. We have tested the effect of dart receipt on the number of sperm stored by once-mated snails, *Helix aspersa*. Snails that were hit by darts stored significantly more sperm than did snails that were missed. Additionally, the effect of the dart was stronger in smaller animals and the number of sperm stored decreased with the shell volume of the recipient. Although larger animals produced larger spermatophores, dart shooting success was not related to the number of sperm transferred. These data suggest a role for dart shooting in post-copulatory sexual selection.

Introduction

Bateman's (1948) principle describes opposing sexual strategies for males and females. Males, whose reproductive success is typically limited by the number of available mates, compete to secure a large number of fertilizations. Females, whose reproductive success is typically limited by the availability of resources, tend to be more discriminating in their choice of mating partners. This simple paradigm becomes paradoxical when applied to simultaneous hermaphrodites because male and female strategies are antagonistic. Many adaptations allow simultaneous hermaphrodites to separate the male and female components of their reproductive success. For instance, the marine flatworm *Pseudoceros bifurcus* tries to hypodermically inseminate its mating partner while avoiding its partner's attempts to do the same (Michiels and Newman 1998). Hypodermic insemination allows a given animal to transfer sperm (mate as a male) without necessarily receiving sperm (mate as a female).

Rather than avoiding being inseminated, pulmonate land snails digest the vast majority of sperm received in a specialized gametolytic gland called the bursa copulatrix (Lind 1973). A small but variable number of sperm escape to the sperm storage organ (spermatheca; Haase and Baur 1995). The factors determining how many sperm are stored by the recipient are unknown. However, one possible influence on sperm storage is a bizarre morphological adaptation named the gypsobellum or 'love dart'.

During the final stage of courtship, helicid land snails attempt to push sharp calcareous darts into their mating partners. The darts are coated with mucus from specialized digitiform glands immediately prior to being shot. Koene and Chase (1998b) demonstrated that mucus extracted from the digitiform glands induced contractions and

conformational changes in the female reproductive tract. These changes were interpreted as speeding the uptake of the spermatophore and sealing off the entrance to the gametolytic bursa copulatrix thereby allowing more sperm to escape digestion and proceed to the storage organ. That is, by successfully shooting a dart, a sperm donor might increase the number of his sperm stored by the recipient without transferring more sperm.

In the present study, we have tested the hypothesis that dart shooting promotes survival of the shooter's sperm within the recipient's female tract (Chung 1987, Adamo and Chase 1996, Koene and Chase 1998b). We selected the helicid snail *Helix aspersa* as a model organism. The exact taxonomic status of this species is controversial; it is variously assigned to the genera *Cornu*, *Cantareus*, and *Cryptomphalus*. Since none of these names has emerged as the standard, we retain the use of *Helix* to be consistent with the existing behavioral literature.

H. aspersa is a simultaneous reciprocal hermaphrodite. During prolonged copulation (6-8 hours; Adamo and Chase 1988), each snail transfers a spermatophore into the partner's bursa tract diverticulum. The sperm escape into the spermoviduct via the long tail of the spermatophore and travel up to the fertilization pouch-spermatheca complex (FPSC; Tompa 1984) where they are stored in blind-ended spermathecal tubules (Lind 1973). The mating system is potentially subject to sperm competition (Parker 1970) since individuals mate with multiple partners between oviposition events (Moulin 1980) and can store viable allosperm for up to 4 years (Duncan 1975). Virtually all individuals possessing darts shoot them during courtship (>97%; Adamo and Chase 1988).

Methods

General methods

Mature and immature garden snails, *Helix aspersa,* were collected from natural populations in Strathmore, California by commercial suppliers. They were isolated upon arrival in small chambers (5 x 5 x 8 cm) and maintained at 18-21°C under a reversed 16L:8D photoperiod. Immature animals were raised to sexual maturity in the laboratory, as recognized by the development of a reflected lip at the shell aperture. Maturation required 3-5 months, during which time the snails were fed calcium carbonate and powdered grains *ad libitum.* The animals were cleaned and fed every 2-3 days. Isolated snails raised to sexual maturity in the laboratory are referred to herein as 'virgins.' Snails that were sexually mature upon arrival and were therefore presumed to have already mated at least once, are referred to as 'experienced'. Since snails do not produce darts until after their first mating (Chung 1986b), we were able to confirm the reproductive status of each animal by observing its mating behavior.

Prior to use in mating trials, mature snails were kept in isolation for at least 10 days to replenish potentially depleted autosperm reserves (Locher and Baur 1999). Mating trials were conducted under a 4-day feeding/ 3-day starvation cycle to maximize sexual proclivity (Adamo and Chase 1991). During the feeding phase, 40-50 snails (the courting group) were placed together in a large Lucite box (36 x 36 x 8 cm) and allowed to court freely. Desired pairs were isolated following the initiation of courtship, and dart shooting behavior was closely monitored.

Scoring dart receipt

Shot darts were assigned to one of two strictly defined categories: 1) miss: the dart was shot but failed to penetrate the recipient, or 2) hit: the dart penetrated over 50% of its length and remained embedded in the recipient throughout copulation. When shot darts fell into either one of these categories, the snails were allowed to continue courting and ultimately trade spermatophores. However, when a dart was shot but did not fall into either of the above classes, the courting snails were immediately isolated and prevented from copulating. In the latter case, the shooter was isolated for 10 days to produce a new dart (Tompa 1984) before it was allowed to court again. The recipient was isolated for 2 days to allow any effect of the received mucus to dissipate. Each animal was permitted to copulate only once, but most animals were involved in multiple courtships (median = 2) before receiving a dart that fell into either of the appropriate categories. Different snails were used in the two studies described below.

Following a successful copulation the heights, lengths and widths of the shells of the snails involved were measured to the nearest 0.1 mm using vernier calipers. These measurements (in mm) were used to calculate shell volume according to the following formula (D. Rogers unpublished data):

$$\text{shell volume } (cm^3) = 3.00 \times 10^{-4} \text{ (height x length x width)} - 0.46$$

Sperm transfer

To study the relationship between dart shooting and sperm transfer, we observed matings between experienced snails only. Accordingly, in this experiment, the courting group consisted of 40-50 experienced animals. The presence of two experienced snails in

each mating pair resulted in reciprocal dart-shooting; every snail shot its partner with a dart during courtship. Pairs were allowed to copulate if at least one of the two shot darts fell into either of the prescribed categories. Otherwise, the snails were separated prior to copulation and isolated as described above before being returned to the courting group.

Mating snails were separated 290 minutes after the initiation of copulation (when filled spermatophores were being transferred; Adamo and Chase 1988) by slowly pulling the shells in opposite directions. Once the spermatophores were expelled by the donors, they were collected and frozen individually at −85°C. At the end of the mating trials, 38 randomly selected spermatophores (19 from each dart shooting class) were disrupted to determine the number of sperm they contained.

Our spermatophore disruption method was modified from the one described by Locher and Baur (1997). We felt it necessary to standardize the duration of disruption across samples so we substituted sonication with a detergent-trypsin protocol adapted from Vindelov et al. (1983). The head and tail were removed from the sperm-containing body of the spermatophore, which was cut open with a fine blade. The contents of the body were scraped out and placed with the shell of the spermatophore into 700 µl of citrate buffer (Vindelov et al. 1983). This suspension was homogenized with a 200 µl pipetman with a tip cut to an internal diameter of 1.6 mm for 5 min before 500 µl of trypsin-EDTA (0.25% trypsin 1:250, 1 mM EDTA·4Na in Hanks balanced salt solution) was added to the mixture. Trituration continued with the same pipette tip for 10 min, at which point 100 µl of Triton X-100 was added. The suspension was then triturated through a tip with an internal diameter of 0.7 mm for 10 min. After adding 200 µl of 4% paraformaldehyde (in 0.1 M PBS), a tip with an internal diameter of 0.5 mm was used to

triturate the suspension for 10 min. The suspension was centrifuged at 4000g for 5 min, the supernatant discarded and the pellet air dried for 10 min. It was then resuspended in 1.0 ml of 4% paraformaldehyde in 0.1 M PBS using a pipette tip with an internal diameter of 0.5 mm. All triturations were performed at a constant rate of 2 sec^{-1}.

The final suspension was diluted 1:9 in 0.1 M PBS and the number of sperm in a sample was counted using an improved Neubauer haemocytometer at a total magnification of 200x. Both intact sperm heads and liberated nuclei were counted.

Images of the spermatophores were captured with a CCTV camera connected to a frame grabber (LG-3, Scion) and spermatophore dimensions were determined using ScionImage. Assuming a cylindrical shape, volume was calculated from the longitudinal area (A) and maximum length along the midline (L) of each spermatophore according to the formula:

$$V = \pi A^2/4L$$

Sperm storage

In a separate experiment, we attempted to identify the effect of dart receipt on the number of sperm stored by once-mated snails. Mating pairs consisted of one virgin and one experienced snail. In each pair, the virgin acted as the sperm recipient and the experienced snail as the dart-shooter/sperm donor. It was necessary to use experienced animals as dart-shooters since unmated snails do not produce darts. Consequently, in this experiment, only the virgin was shot with a dart. We conducted mating trials as described above but virgins were included in the courting group under a ratio of one virgin to three experienced snails. The total size of the courting group remained 40-50 individuals.

Following successful dart shooting and copulation, 'virgins' were isolated for 7 days to allow the allosperm to reach the spermatheca. At the end of 7 days, they were anaesthetized by injection of 2 ml isotonic $MgCl_2$ (97.4 mM $MgCl_2$, 5 mM Tris·HCl). Shells were removed and the FPSCs dissected out. Each FPSC was fixed in 1.0 ml 4% paraformaldehyde for 60 min then transferred to a 1.0 ml solution of 9 μM Hoechst 33342 in 4% paraformaldehyde for 180 min. This protocol left the sperm heads inside the FPSC intensely labeled and the spermathecal epithelium lightly labeled.

Stained FPSCs were washed in 0.1M PBS for 5 min, embedded in 7% agar, and serially sectioned longitudinally at 100 μm on a Vibratome. Sections were dehydrated in an ascending ethanol series, cleared in methyl salicylate and mounted in Permount. Sperm were counted under UV fluorescence (Leitz D-cube on a Leitz Dialux 20 epifluorescent microscope) using a Leitz NPL Fluotar 40x objective with a numerical aperture of 1.30 under oil immersion at a total magnification of 400x. The depths of the profiles (~1-2 μm) relative to the section thickness made the risk of duplicating counts negligible. Counting sperm in sections allowed us to distinguish between allosperm stored in the spermathecal tubules and autosperm in the fertilization pouch. Since the walls of the FPSC were lightly stained, we were able to reconstruct its anatomy to distinguish the spermathecal tubules from the fertilization pouch. Moreover, spermathecal allosperm are stored with their heads pointed towards the tubular epithelium and their tails extending out towards the opening to the fertilization pouch. Autosperm within the pouch are recognizable by their lack of orientation (Lind 1973). Slides were coded and all counts were performed at the end of the mating trials without prior knowledge of the source of the tissue.

Statistical analysis

Statistical analyses were performed using Systat 8.0 (SPSS). Factors affecting the number of sperm transferred and the number of sperm stored were evaluated using generalized linear model analysis of covariance (GLM ANCOVA), a multiple regression-based method. Categorical variables were included in the linear models through effects coding. Backward elimination of statistically non-significant factors (all with $P > 0.40$) was employed to obtain the most parsimonious models (Sokal and Rohlf 1969). All reported P values are two-tailed.

Results

Spermatophore volume and sperm number

Linear regression of the number of sperm transferred on spermatophore volume (Fig. 1) revealed a highly predictive relationship ($r^2 = 0.91$, $F_{1,36} = 348.8$, $P<0.001$). This allowed us to estimate the number of sperm contained in the remaining spermatophores according to the linear formula:

$$\text{sperm number } (\text{x}10^6) = 1.093(\text{spermatophore volume}) - 1.573$$

Analysis of the residuals provided no evidence of any difference in the density of sperm in the spermatophores produced by dart shooters that hit their partners or dart shooters that missed their partners (two-tailed t-test: $df = 36$, $t = 1.18$, $P = 0.244$), warranting the use of the above equation for both types of shooters.

Sperm transfer

We collected spermatophores from 64 animals (from a total of 43 pairs) whose shots fell into one of the two prescribed categories. These snails transferred between 1.05×10^6 and 13.73×10^6 sperm with the exception of a single animal that produced an empty spermatophore. The mean ± SD number of sperm transferred was $5.56 \times 10^6 \pm 2.88 \times 10^6$ (n = 64). GLM ANCOVA revealed that the sperm donor's shell volume had a significant positive effect on the number of sperm transferred (Table 1, Fig. 2). Despite a strong trend towards successful dart shooters transferring fewer sperm than unsuccessful shooters (least squares mean ± SE, missed = $6.29 \times 10^6 \pm 0.03 \times 10^6$, n = 29; hit = $5.06 \times 10^6 \pm 0.03 \times 10^6$, n = 35), we failed to detect a statistically significant effect of dart shooting success (Table 1). Since each snail in a mating pair acted as both sperm donor and sperm recipient, we included mating pair as a blocking variable in an early model. Mating pair had no significant effect on the number of sperm transferred and was therefore dropped from the model through backward elimination, thus allowing each snail to be treated as an independent data point. The candidate independent variables dart receipt category, shell volume of the sperm recipient, number of sperm received, and all possible two-way interactions were also found to have no significant effect on the number of sperm transferred and were likewise eliminated from the model. All dropped variables had $P > 0.40$.

In total, 22 animals were excluded from the above analysis because their shot darts did not fall into either of the prescribed categories. When all collected spermatophores (n=86) were included in an expanded data set, we once again failed to find any effect of mating pair, recipient shell volume, the number of sperm received, or any interaction on

the number of sperm donated using multiple regression analysis. Regression of the number of sperm transferred on donor shell volume again revealed a significant effect ($b = 0.85 \times 10^6$ sperm/cm^3, $r^2 = 0.14$, $F_{1,84} = 13.33$, $P < 0.001$).

Sperm storage

Of the 39 snails examined, 37 were found to be storing allosperm in their spermathecae. The number of sperm stored by once-mated animals ranged from 0 to 2986, with a mean ± SD of 1425 ± 879 (n = 39). This mean corresponds to roughly 0.025% of the mean number of sperm transferred. Since sperm were invariably destroyed during spermatophore disruption, this number is almost certainly an overestimate of the proportion of transferred sperm that reach the storage organs. However, our estimate agrees with Lind's (1973) approximation that 0.1% of donated sperm escape digestion in the closely related species *Helix pomatia*.

The effects of dart receipt, recipient shell volume, donor shell volume, and all possible two-way interactions on the number of sperm stored were investigated by GLM ANCOVA (Table 2, Fig. 3). Dart receipt had a strong positive effect on the number of sperm stored. Snails penetrated by their partners' darts stored 116% more sperm than snails that were not penetrated by darts (least squares mean ± SE, missed = 917 ± 41, n = 21; hit = 1983 ± 54, n = 18). Furthermore, the body size of the sperm recipient - represented by shell volume - was negatively associated with the number of sperm stored. The marginally significant interaction observed between dart receipt and shell volume indicates that the effect of the dart is stronger in small animals than in large ones. No significant effect of donor body size or any other interaction was detected, and these terms were dropped from the model by backward elimination.

Note that the squared multiple correlation coefficient of the linear model used in the analysis was only 0.543, suggesting that other factors not examined in this study may influence the number of sperm stored after a single mating.

Discussion

We have established an association between dart receipt and sperm storage; once-mated snails hit by darts stored significantly more sperm than once-mated animals of equal size that were not hit by darts. However, it is possible that the observed effect of dart receipt on sperm storage is due to an association between dart shooting and some other factor - the most plausible being the number of sperm transferred - rather than any direct physiological effect of the dart. For instance, in both the capercaillie *Tetrao urogallus* (Mjelstad 1991) and the Trinidadian guppy *Poecilia reticulata* (Matthews et al. 1997) male courtship behavior is related to ejaculate volume. In these species, male display rates signal 'direct fertility benefits' to females. However, we found no evidence that successful dart shooters transfer more sperm to their mating partners than do unsuccessful shooters. That is, dart receipt is an unreliable indicator of ejaculate volume. Actually, we found a strong, but not statistically significant, trend in the opposite direction; unsuccessful shooters transferred more sperm than successful ones. This trend, if borne out by future investigation, would suggest that unsuccessful shooters might attempt to compensate for the reduction in the proportion of their ejaculate stored by transferring more sperm. In any case, it is unlikely that the observed relationship between dart receipt and sperm storage is the indirect result of a correlation between dart shooting success and a third unknown factor.

Leonard (1992) argued that dart shooting signals the shooter's intention to mate as a male, not only to indicate direct fertility benefits, but also to stimulate the partner to reciprocate in kind. We found no evidence that dart receipt affects the number of sperm donated. Furthermore, we found that 83 of 84 copulating snails fulfilled the male role. Baur et al. (1998) found a similar number (91 of 92 snails) in the copse snail *Arianta arbustorum*, a species where the frequency of dart shooting is only about 30% (Baminger et al. 2000). Consequently, it seems that neither dart shooting nor dart receipt influences a snail's willingness to donate sperm.

The hypothesis of a direct physiological effect of dart receipt on the number of sperm stored is well supported. Koene and Chase (1998b) demonstrated *in vitro* that extracts of the mucus-producing digitiform glands close off the entrance to the gametolytic bursa copulatrix where the bulk of the donated allosperm are destroyed. Furthermore, we have observed that the effect of dart receipt is stronger in small animals than in large animals - likely the result of a dilution of the digitiform mucus within the haemocoel of larger recipients.

Successful dart shooters stand to benefit in two separate ways from the increased number of sperm stored by their mating partners. First, a large number of stored sperm can be used to fertilize a large number of eggs resulting in high male reproductive success. Chen and Baur (1993) demonstrated that sperm from a single mating can be used by *A. arbustorum* to fertilize as many as 11 consecutive clutches, even extending into a second breeding season. Thus, in the absence of sperm competition, successful dart shooters will enjoy higher male reproductive success than unsuccessful shooters. Non-competitive mating may have been an important influence in the evolution of dart shooting since low population density and the associated low mating rate have been

proposed as factors contributing to the origin and maintenance of hermaphroditism (Ghiselin 1969). However, the promiscuity of land snails generally results in clutches of mixed paternity. Indeed, Murray (1964) estimated the average number of fathers per clutch to lie between two and five for the black-lipped snail *Cepaea nemoralis*. Therefore, sperm from different males usually mix within the female reproductive tract, either in the spermatheca during storage or in the fertilization pouch during oviposition, resulting in sperm competition. However, the mechanism of sperm utilization employed by *H. aspersa* is not presently understood. If fertilization occurs through a 'fair raffle' (Parker et al. 1990) where fertilization success is decided by the proportional representation of each male's sperm in the spermatheca, successful dart shooters would enjoy greater male reproductive success in competitive matings than unsuccessful shooters, assuming that the effect of dart receipt observed in this study extends beyond the first mating.

Recent results from our laboratory (Landolfa et al. in press) provide evidence that the observed effect of dart shooting also occurs in multiply mated animals. Dart shooting influenced paternity ratios in the clutches of twice-mated snails, but only the second male's shot had a statistically significant effect. If both males shot 'well', the second male fathered 48% of the eggs in the clutch. However, if the second male shot 'well' and the first male shot 'poorly', the second male fathered 60% of the eggs. These numbers correspond well with the findings of the present study. Since snails stored 116% more sperm from successful shooters than from unsuccessful shooters, under the fair raffle principle we would expect a successful shooter to fertilize 68% of the eggs in a competitive mating against an unsuccessful shooter.

It has been suggested that the dart serves as a basis for mate choice either as a nuptial gift of calcium or as an indicator of the shooter's innate quality (Charnov 1979).

While the first hypothesis has been tested and rejected (Koene and Chase 1998a), the second remains a viable possibility. Without knowledge of the net costs and benefits of dart shooting and receipt it is very difficult to determine if snails increase the number of sperm stored with dart receipt through cryptic mate choice or if this response is due to manipulation of the recipient by the shooter (Adamo and Chase 1996).

Greeff and Michiels (1999a) provided a basis for further insight into the advantages of successful dart shooting. They demonstrated that sperm digestion coupled with sperm competition could lead to an intersexual arms race with the male component evolving to transfer larger ejaculates and the female component evolving to digest more of the received sperm. Escalation of the arms race could potentially lead to a point where Bateman's principle would collapse. That is, the required resource allocation to the male function could approach or even surpass the amount allocated to the female function (but see Locher and Baur 2000). By reducing the amount of sperm digested by the recipient, dart shooting allows donors to transfer smaller ejaculates. Dart shooting would be economical so long as the dart is less expensive to produce than the excess sperm.

The number of sperm transferred in a single mating by *H. aspersa* ($5.56 \times 10^6 \pm 2.88 \times 10^6$) is comparable to the number for *A. arbustorum* ($2.21 \times 10^6 \pm 0.86 \times 10^6$; Baur et al., 1998), the only other helicid species examined to date. The large ejaculates of pulmonate snails, relative to the number of sperm that reach the storage organ, are consistent with Greeff and Michiels' (1999a) prediction of an evolutionary arms race resulting from the interaction between sperm competition and sperm digestion. Our observation that larger snails produce larger spermatophores can likely be explained by allometry. Roughly 99.98% of transferred sperm were immediately digested by the

recipient in *H. aspersa*, indicating that the female tract is extremely hostile towards allosperm. If, as proposed by Short (1981), larger female tracts are more hostile to sperm than smaller tracts, then we might attribute the negative effect of recipient body size on the number of sperm stored to the greater hostility of the tracts of larger recipients. Since donors do not appear to tailor their spermatophores to the size of the recipient, we would expect fewer sperm to reach the spermathecae of larger snails. The greater hostility of the reproductive tracts of larger animals may be adaptive, allowing these animals to derive more energy from digested sperm, or to eliminate sperm from low quality donors (Birkhead et al. 1993).

The selective pressures of pre-copulatory mate choice are relaxed in simultaneous hermaphrodites (Greeff and Michiels 1999b). Indeed, there is no evidence that helicid land snails discriminate between potential mates based on shell volume (Baur 1992) or relatedness (Baur and Baur 1997). However, our findings on the effect of dart shooting in *H. aspersa* indicate that post-copulatory sexual selection might play an important role in the mating systems of simultaneous hermaphrodites.

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Table 1. GLM ANCOVA model ($R^2 = 0.183$) for factors affecting the number of sperm transferred by copulating snails (n = 64).

Factor	*df*	*MS*	*F*	*P*
Donor shell volume	1	72.87	11.28	0.001
Dart shooting success	1	21.64	3.35	0.072
Error	61	6.46		

Table 2. GLM ANCOVA model for factors affecting the number of sperm stored by once-mated snails (n = 39). The term dart receipt x recipient volume indicates a statistical interaction.

Factor	*df*	*MS*	*F*	*P*
Dart receipt category	1	4.61×10^6	12.18	0.001
Recipient shell volume	1	5.00×10^6	13.21	0.001
Dart receipt x recipient volume	1	1.56×10^6	4.13	0.050
Error	35	3.78×10^5		

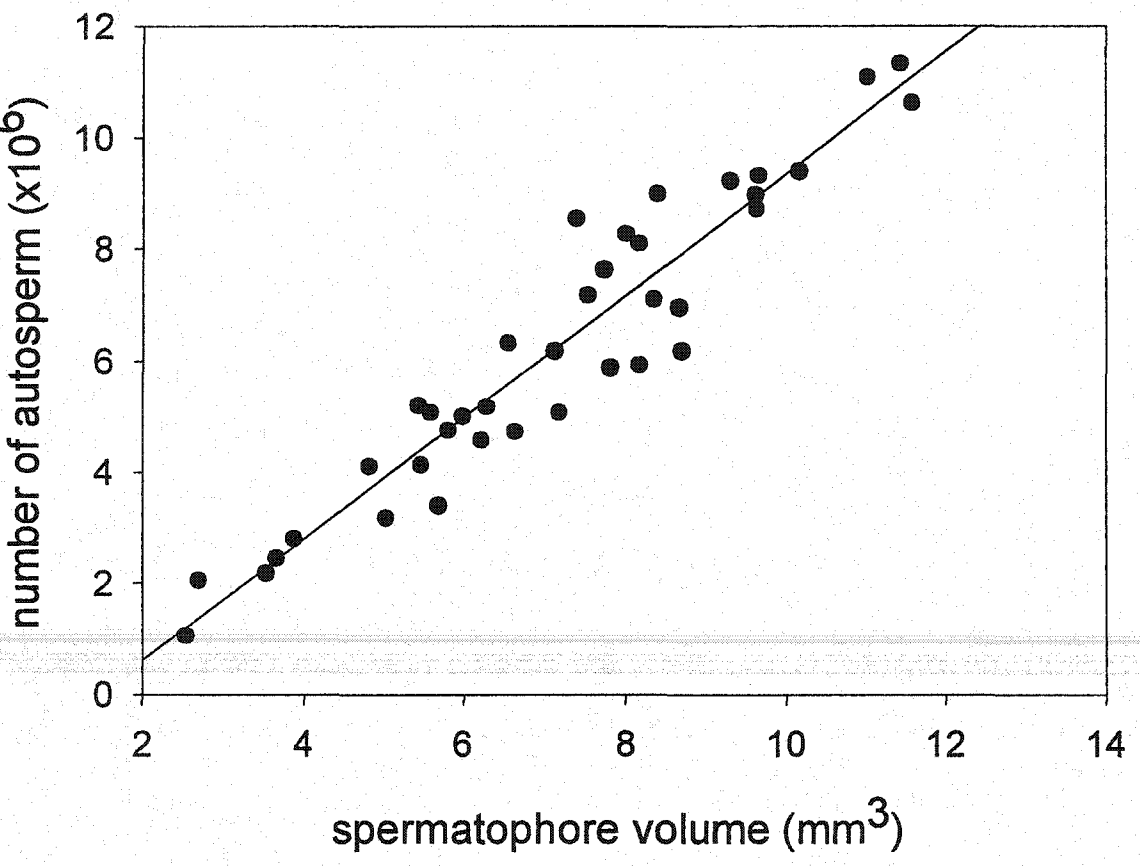

Fig. 1 The relationship between the number of sperm transferred and spermatophore volume.

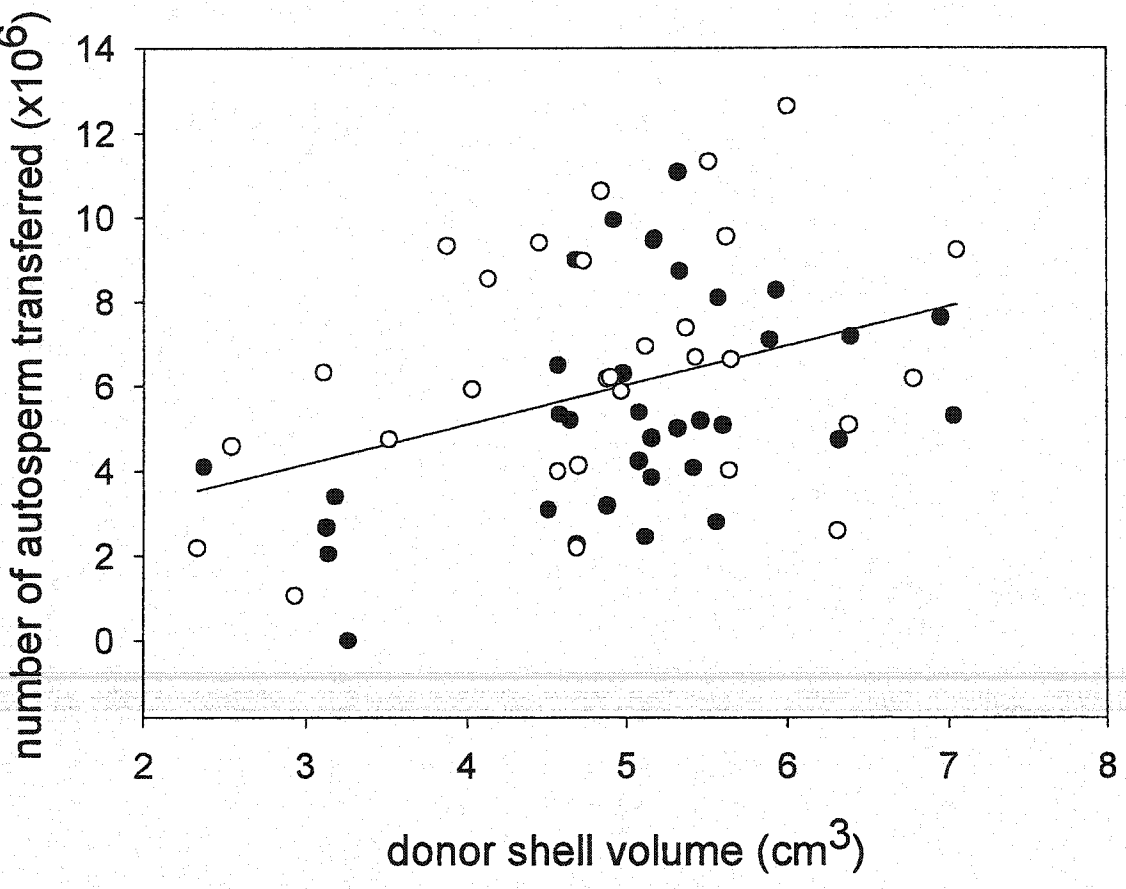

Fig. 2 The relationship between the number of sperm transferred and donor body size. Open circles: shot dart missed recipient. Closed circles: shot dart hit recipient.

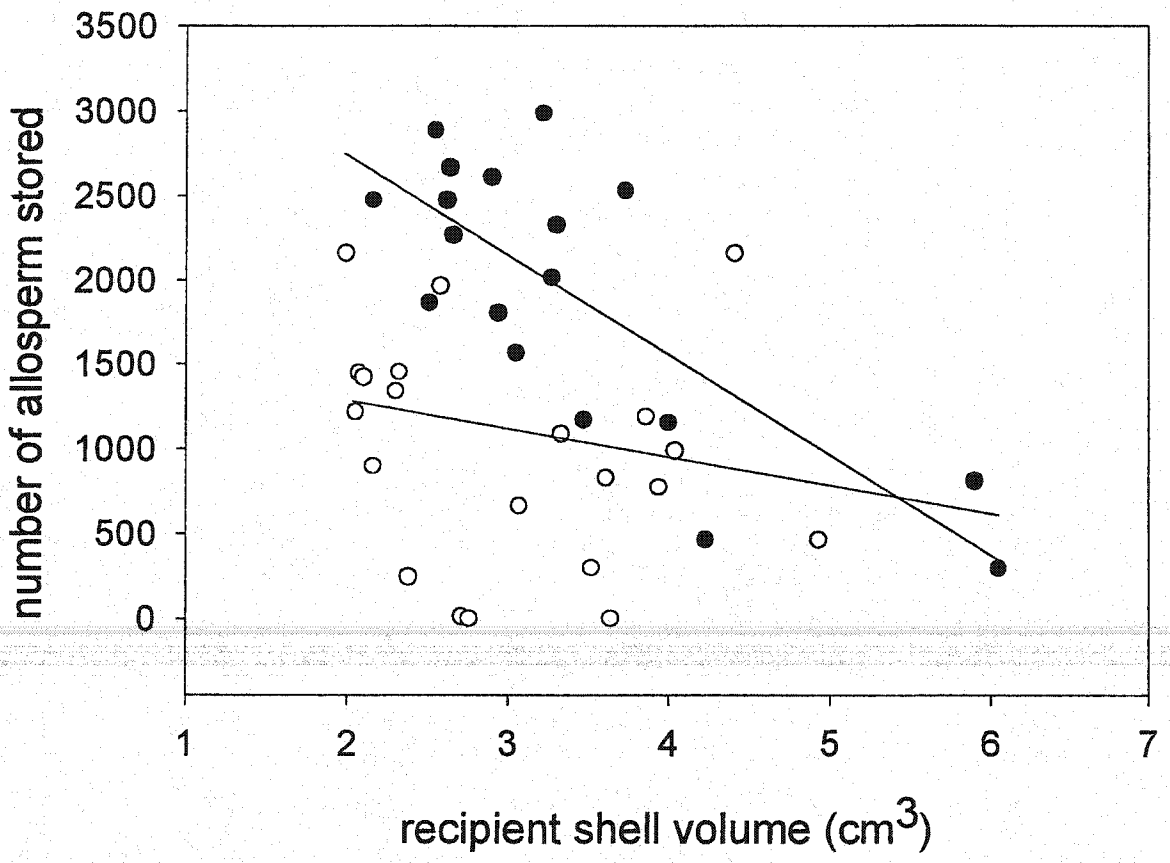

Fig. 3 The effect of dart receipt on the number of sperm stored by recipients of different volumes. Open circles: shot dart missed recipient. Closed circles: shot dart hit recipient.

Chapter 3

DETERMINANTS OF PATERNITY IN THE GARDEN SNAIL *HELIX ASPERSA*

In this chapter, I test the second prediction of the sperm loading hypothesis : that the biased storage of sperm described in Chapter 2 results in higher paternity scores for successful shooters, compared to unsuccessful shooters, in competitively fertilized clutches. Additionally, I attempt to identify other factors influencing the distribution of paternity scores between competing sperm donors. Finally, I propose a novel mechanism to explain the observed patterns of sperm utilization in *H. aspersa*.

Abstract

Despite their importance to the understanding of sexual selection in simultaneous hermaphrodites, the factors influencing the outcome of sperm competition in these organisms are generally unknown. We have investigated the effect of dart-shooting, mating order and several other predictors on the proportion of offspring fathered by second sperm donors (P_2) in twice-mated garden snails, *Helix aspersa*. While paternity ratios were biased towards the first donor (mean $P_2 = 0.32$), the magnitude of this advantage was dependent upon which of the two donors successfully darted the recipient. Mean P_2-values increased from 0.21 when the recipient was hit by the first donor to 0.46 when it was hit by the second. Furthermore, the effect of the dart was more pronounced in the clutches of smaller recipients. From these results, and observations of live sperm in the storage organs, we propose a novel mechanism to explain the detected pattern of sperm utilization in helicid snails.

Introduction

Despite a strong theoretical basis (Charnov 1996, Greeff and Michiels 1999a) empirical studies of sperm competition (Parker 1970) in simultaneous hermaphrodites are rare. Moreover, the reciprocal nature of copulation in many of these organisms has engendered a view of their mating systems as being largely cooperative (Leonard 1990). However, reciprocation does not imply an absence of conflict. It indicates that, rather than being restricted to the male half of the population, sperm competition engages every individual hermaphrodite in a contest for paternity. Although the determinants of success or failure in this conflict remain poorly understood, sperm competition between simultaneous hermaphrodites is likely a powerful selective agent shaping morphology, physiology and behavior (Michiels 1998).

In the present study, we have attempted to identify factors influencing sperm utilization in the simultaneously hermaphroditic land snail *Helix aspersa*. While only intermating interval has been identified as a determinant of paternity in snails (Baur 1994), other factors are suggested by the literature. Several studies have provided evidence of first-male sperm precedence in gastropod molluscs. High paternity scores have been assigned to early sperm donors after multiple subsequent matings in both the nudibranch *Phestilla sibogae* (Todd et al. 1997) and the marine prosobranch *Littorina obtusata* (Paterson et al. 2001). Of more relevance to the current study, non-significant trends toward first-male precedence have been reported in the terrestrial snails *Arianta arbustorum* (Baur 1994; $P_2 = 0.34$) and *Helix aspersa* (Landolfa et al. in press; $P_2 = 0.40$).

The "love dart" has also been proposed as a factor promoting paternal reproductive success (Koene and Chase 1998b). During the final stage of courtship,

helicid land snails attempt to push sharp calcareous darts into their mating partners. Once-mated snails struck by darts store roughly twice as many sperm as those avoiding their partners' shots (Rogers and Chase 2001). Mucus derived from specialized digitiform glands coats the dart and induces conformational changes in the recipient's reproductive tract, likely closing off the entrance to the gametolytic bursa copulatrix where the vast majority of transferred sperm are digested (Koene and Chase 1998b), allowing more sperm to escape to the storage organs in darted recipients. A preliminary study of the influence of dart shooting on paternity ratios in the clutches of twice-mated snails found only a marginally significant effect of the second donor's dart and no significant effect of the first donor's dart (Landolfa et al. in press).

We have investigated the influence of dart shooting, mating order, intermating interval and several other factors on paternity ratios in the offspring of twice-mated snails. Based on the results of this analysis and observations of allosperm in the spermathecae, we propose a novel mechanism to explain the observed pattern of sperm utilization in *H. aspersa.*

H. aspersa is a simultaneous reciprocal hermaphrodite. During prolonged copulation (6-8 hours; Adamo and Chase 1988), each snail transfers a spermatophore containing between 1×10^6 and 14×10^6 sperm (Rogers and Chase 2001) into its partner's bursa tract diverticulum. While the vast majority of these sperm are digested in the bursa copulatrix, a small number escape into the spermoviduct via the long tail of the spermatophore and travel up to the fertilization pouch-spermatheca complex (FPSC; Tompa 1984). Roughly 0.025% of the transferred sperm are stored in the blind-ended spermathecal tubules of the FPSC (Rogers and Chase 2001). Fertilization coincides with oviposition which general occurs only once per breeding season (Madec et al. 1998). The

mating system is potentially subject to sperm competition since individuals mate with multiple partners (1-6; Madec et al. 1998) between oviposition events and can store viable allosperm for up to 4 years (Duncan 1975). Virtually all individuals possessing darts shoot them during courtship (>97%; Adamo and Chase 1988). The exact taxonomic status of this species is controversial; it is variously assigned to the genera *Cornu*, *Cantareus*, *Cryptomphalus* or *Helix*. Since none of these names has gained consensus status, we retain the use of *Helix* to be consistent with the existing literature.

Methods

General methods

Sexually mature garden snails, *Helix aspersa*, were collected by commercial suppliers from three geographically distant natural populations near the Californian cities of Strathmore, Fresno, and Long Beach, as well as from one natural population in Argentina. The approximate linear distances between the Californian populations ranged from 96 km to 364 km.

Upon arrival in the laboratory snails were isolated in small chambers (5x5x8 cm) and, in most cases, stored under hibernation-inducing conditions (24-hour darkness at 10°C) for 3-6 weeks. Once aroused from hibernation, isolated snails were maintained at 18-21°C under a 16 h light:8 h dark photoperiod. They were cleaned and fed ad libitum a diet of chicken feed and powdered grains supplemented with calcium carbonate. Prior to use in mating trials, snails were isolated for a further 2 weeks and individually marked by gluing a small numbered plastic tag (queen marking kit, The Bee Works, Orillia, Canada)

to the shell. Using different colored tags to identify each population, we marked a total of 96 snails from each collection site.

Mating trials

Trials were designed to obtain recipient snails mated with two different sperm donors. Appropriate matings met two requirements: 1) the two donors and the recipient all originated from different populations and 2) the recipient was hit by the dart of one donor and missed by the dart of the other. Donors from different populations were selected for two reasons. First, since land snails exhibit very low levels of within-population genetic diversity and comparatively high levels of between-population genetic diversity (Arnaud et al. 2001), using potential fathers from genetically unrelated populations increased our ability to distinguish between the two donors in question as well as any previous donors (which would be genetically similar to the recipient). Second, if the dart is subject to antagonistic co-evolution (i.e. if stronger effects of the dart are selected for in the male component and resistance to the dart is selected for in the female component) then we would expect females to be most resistant to the darts of sympatric donors (see Knowles and Markow 2001). By using allopatric shooters, we hoped to capitalize on reduced female resistance to observe a stronger effect of dart receipt.

Mating trials were conducted under a 4-day feeding/3-day starvation cycle to maximize sexual proclivity (Adamo and Chase 1991). During the feeding phase, approximately 10 snails from each population were placed in each of several large Lucite boxes (36x36x8 cm), provided with sliced carrots, and allowed to court freely. Desired pairs were isolated following the initiation of courtship, and dart-shooting behavior was closely monitored. Courtships were interrupted if the courting snails were from

inappropriate populations, or if either of the snails was shot inappropriately. Shot darts were assigned to one of two strictly defined categories (Rogers and Chase 2001): 1) miss - the dart was shot but failed to penetrate the recipient or 2) hit - the dart penetrated over 50% of its length and remained embedded in the recipient throughout copulation. When a dart was shot inappropriately (i.e. in the same category as the recipient's first partner or in neither of the defined categories), the shooter was isolated for 10 days to produce a new dart (Tompa 1984) before it was allowed to court again. The recipient was isolated for 2 days to allow any effects of the received mucus to dissipate.

Following successful copulation, mated snails were isolated for 8 days to allow allosperm to reach the spermathecae (Lind 1973) and to restore depleted autosperm reserves (Locher and Baur 1999). Mated snails were subsequently either returned to the pool of courting snails (after their first mating) or placed in egg-laying chambers (after their second mating). Isolated snails were prevented from laying prior to completion of their second mating by depriving them of soil substrate. Dead and moribund snails discovered during the course of the mating trials were immediately frozen at -20°C, as were successful egg-layers.

Egg-laying

Appropriately twice-mated snails were placed in individual chambers (8 cm x 14 cm) lined with 5 cm of a 3:1 mixture of potting soil and powdered oyster shell. These chambers were maintained under a 14 h light:10 h dark photoperiod with a 'light' temperature of 22-23°C and a 'dark' temperature of 18-19°C. High humidity was maintained at all times. Candidate egg-layers were subjected to a 2-day feeding/5-day starvation cycle to promote oviposition.

Chambers were checked daily for eggs, which were allowed to hatch. Offspring were fed and kept active until the shell lengths of the largest animals reached 1 cm, at which point they were forced to aestivate. All offspring were starved for at least 7 days prior to genotyping.

The heights, lengths, and widths of the shells of all successful egg-layers and their mating partners were measured to the nearest 0.1 mm using vernier calipers. These measurements (in millimeters) were used to calculate shell volume according to the following formula (D. Rogers, unpublished data):

$$\text{shell volume } (cm^3) = 3.00 \times 10^{-4}(\text{height} \times \text{length} \times \text{width}) - 0.46$$

Paternity determination

Following egg-laying, samples of the hepatopancreases of the parental snails were prepared for electrophoretic analysis. After rinsing in distilled water, samples were homogenized in 750µl distilled water and slowly frozen at -20°C to encourage cell disruption, then stored at -85°C. Randomly selected offspring (n=30) from each clutch were prepared using a similar protocol, only entire snails were each homogenized in only 300µl distilled water. Immediately prior to electrophoresis, samples were thawed, briefly vortexed and centrifuged at 10,000 rpm for 15 minutes. The resulting supernatants were run on horizontal starch gels as described by Murphy et al. (1996). All 3 parents and up to 27 offspring were included on each gel. Based on previous results (Landolfa et al. in press), we used the amine citrate (morpholine) pH 6.1 buffer system described by Clayton and Tretiak (1972). We initially tested 12 loci: ADH, MDH, LDH, IDH, EST, SOD, PER, ALP, ACP, CAP, AAT, and CAT but obtained the best combination of staining quality

and allelic diversity using CAP (EC 3.4.11.1; two distinct loci), AAT (EC 2.6.1.1; single locus), and CAT (EC 1.11.1.6; single locus). In all clutches analyzed, the genotypes of the first and second mated male were completely distinct for at least one locus. In 29 of the clutches, the two male genotypes were distinct for at least two loci.

Observations of stored allosperm

Fertilization pouch spermatheca complexes were removed from snails 7-72 days after mating by cutting the ducts connecting to the seminal vesicle and the spermoviduct. Prior to dissection snails were either anaesthetized by injection of 2 ml isotonic $MgCl_2$ (97.4mM $MgCl_2$, 5 mM Tris·HCl) or left unanaesthetized. Each FPSC was placed in a depression slide with a drop of either snail saline (Kerkut and Meech 1966), phosphate buffered saline, or snail haemolymph and viewed under oil immersion at 1000x total magnification. Observations were initiated within 5 minutes of the start of dissection. Video sequences were captured with a CCTV camera connected to a frame grabber (LG-3, Scion) and recorded on VHS tape. Snails used for videomicroscopy were not involved in the mating trials described above.

Statistical analysis

Statistical analyses were performed using Systat 8.0 (SPSS). Paternity data were analyzed using generalized linear model multiple regression. The categorical variable "dart shooting order" was included in the model through effects coding. Backward elimination of statistically non-significant factors ($P > 0.3$) was employed to obtain the most appropriate model. All reported P-values are two-tailed.

Results

Mating trials

We observed approximately 500 courtships (roughly 1000 separate dart shooting events) resulting in a total of 108 appropriate reciprocal matings. From these observations, we obtained 64 appropriately twice-mated snails, 38 (59%) of which produced clutches of eggs. Since 3 clutches (8%) failed to hatch, 35 broods were available for paternity determination.

Each egg-layer was shot with a dart in only one of its two matings; in 19 cases the first donor shot successfully while in the remaining 16 cases the second donor was successful. The time interval between the two matings for each egg-layer ranged from 8 to 77 days with a mean ± SD of 27.2 ± 16.2 days.

We detected third fathers in 17 (49%) of the 35 clutches. The proportion of offspring assigned to third fathers ranged from 0.00 to 0.52 with a mean ± SD of 0.11 ± 0.15. These offspring were not included in the calculations of second-donor paternity (P_2) scores (i.e. $P_1 + P_2 = 1.00$). Consequently, the number of offspring used to determine P_2 scores ranged from 13 to 27 with a mean ± SD of 21.0 ± 3.7.

To normalize the distribution of paternity proportions, P_2-values were transformed using the arcsine transformation ($p' = \sin^{-1}p^{½}$; Zar 1996). The arcsine transformation was preferred over the logit transformation as the data set contains several 0 values. The transformed mean ± SD P_2-value for the 35 clutches examined was 0.60 ± 0.29, which corresponds to an untransformed mean P_2-value of 0.32. This value is significantly lower

than 0.5 (one sample t-test on transformed data, $t = -3.912$, $df = 34$, $p<0.001$), indicating that paternity is biased in favor of first donors.

We employed backwards elimination to obtain the generalized linear model which best accounts for the observed variance in the transformed P_2-scores. Our preliminary model included the following candidate predictors: a constant, order of dart receipt, maternal shell volume, the interaction between order of dart receipt and maternal shell volume, the proportion of progeny assigned to outside fathers, intermating interval (inverse transformed to normalize the distribution) and the difference in shell volume between the first and second fathers. Difference in paternal shell volumes was calculated as the shell volume of the first donor minus the shell volume of the second donor. The inclusion of maternal shell volume and inter-mating interval failed to improve the model more than would be expected by chance alone (in both cases $P > 0.3$), so both candidate predictors were dropped. The improvements in the model associated with the inclusion of two predictors (the proportion of offspring sired by third fathers and the difference between the first and second father's shell volumes) were marginally significant (0.085 and 0.051 respectively; Table 1). Since our purpose is partially to explore the factors influencing paternity, we included these predictors in the final model. However, these probabilities are large in absolute terms and problems of capitalization on chance associated with stepwise regression require that they be viewed skeptically. The coefficient associated with paternal size difference was negative (Table 1), indicating a trend for a larger donor to father a greater proportion of the progeny than its smaller counterpart. Additionally, the coefficient for the proportion of offspring assigned to third fathers was positive (Table 1), indicating a reduction in the first donor's advantage with increasing contributions from outside fathers.

The effects of both dart-shooting order and the interaction between maternal shell volume and dart-shooting order on the transformed P_2-values were highly significant (Table 1). As depicted in Fig. 1, successful dart shooting increased the proportion of offspring fathered by the successful shooter. When the first sperm donor shot successfully (and the second missed) it fathered, on average, 79% of the offspring while the second donor fathered the remaining 21% (least-squares mean ± SE of transformed P_2-values = 0.47 ± 0.03). In contrast, when the second donor shot successfully (and the first missed), it more than doubled its reproductive success, fathering an average of 46% of the offspring (least-squares mean ± SE of transformed P_2-values = 0.75 ± 0.03) and reducing the first donor's paternity to only 54%. However, the effect of the dart was dependent on the size of the recipient, with the greatest advantage of successful shooting associated with the smallest recipients (Fig. 1).

Observation of stored allosperm

Allosperm in the storage organs were observed to be active at all post-copulatory intervals examined (7-72 days after mating). Most heads were embedded in the epithelium at the blind-ends of the tubules although some were present in the lumen amidst the tails. The tails beat in a synchronous wave that was directed towards the entrance of the tubule (Fig. 2). Individual sperm heads could be seen moving within the tubules (Fig. 2) indicating that flagella are not passively undulating in response to the beating of cilia lining the tubule walls.

We adopted several measures to minimize the possibility that manipulation of the FPSC was activating the sperm. The observed activity was dependent on neither the

medium bathing the FSPC (snail saline, phosphate buffered saline, or snail haemolymph) nor on the use of an anaesthetic prior to dissection. Moreover, physical manipulation of the FPSC was limited to freeing the organ from the snail and placing it in a depression slide. Similar actions failed to activate autosperm in the seminal vesicle or allosperm in spermatophores.

Discussion

We have identified dart-shooting success and mating order as determinants of paternity in the garden snail *Helix aspersa*. While the first donor exhibited a competitive advantage, the magnitude of this advantage was contingent upon which snail successfully darted the recipient. Indeed, successful shooting by the second male negated the advantage of mating first, particularly in smaller recipients. The dependence of the efficacy of the dart on recipient shell volume can likely be attributed to dilution of the digitiform mucus in the haemocoel of larger recipients (Rogers and Chase 2001).

Although the effect of successful dart shooting is clear, we cannot conclude from these data alone whether the shots of both donors affect paternity or if the observed effect is mediated by the dart of either the first or second shooter alone. However, previous studies suggest active roles for both. Rogers and Chase (2001) demonstrated a large increase in the number of sperm stored by once-mated snails struck by their partners' darts. Thus, the first donor stands to fertilize a higher proportion of the recipient's eggs if its shot is successful. Landolfa et al. (in press) found a marginally significant increase in the proportion of eggs fathered by the second donor when its shot hit. The failure of Landolfa et al. to detect a significant effect of the first male's dart or mating order on P_2 can likely be attributed to a lack of statistical power resulting from small sample sizes.

Moreover, the classification of shots as 'good' or 'poor' was somewhat arbitrary, increasing the level of noise in the data. Sample sizes in the current study were 3-4 times larger than those in Landolfa et al.'s analysis, and the difference between successful and unsuccessful shots was maximized.

First-male sperm precedence is uncommon across most taxa and the underlying mechanisms are poorly understood. Consistently low P_2 values are generally attributed to one of four causes: post-copulatory mate guarding, the use of mating plugs, sperm stratification or spermathecal filling. While some gastropods guard their mates following successful copulation (Bradshaw-Hawkins and Sander 1981) and others produce mating plugs (van Duivenboden and ter Maat 1988), helicids engage in neither activity. Stratification of stored allosperm from different donors in 'conduit' spermathecae, which provide a direct connection between the sites of insemination and fertilization, is thought to result in first-male precedence (Austad 1984). However, the sperm storage tubules of helicid snails are blind-ended (which, according to this model, would promote last-male precedence) and there is no evidence of sperm stratification in these animals.

The fourth hypothesis – spermathecal filling (Retnakaran 1974) - seems initially plausible as the capacity of any sperm storage organ is finite. Individual snails possess relatively few thin tubular spermathecae (4-6 in *H. aspersa*; Brisson et al. 1977) and the majority of sperm are stored in a single tubule (Baminger and Haase 1999). Allosperm are stored with their heads in tight contact with the spermathecal epithelium, usually at the bulbous blind-ends of the tubules (Bojat et al. 2001). Todd et al. (1997) argued that the limited availability of these epithelial 'slots' might result in first-male precedence. The first donor's sperm would fill the majority of slots leaving only a small number of storage positions available to the second donor resulting in biased storage favoring the

first male. However, this hypothesis predicts that the spermatheca would fill to capacity after only two matings and subsequent partners would not contribute to the allosperm reserves. In contrast to this prediction, natural helicid snail populations exhibit high levels of multiple paternity (2-5 fathers per brood; Murray 1964). Additionally, the number of sperm stored after a single mating, particularly by large snails, is insufficient to fill the majority of epithelial slots (Rogers and Chase 2001).

We propose a novel mechanism to account for the observed pattern of sperm utilization in *H. aspersa* based on the activity of allosperm in the spermathecae. Activity of stored allosperm has been reported in many taxa (tardigrades, De Zio and Gallo 1975; gastrotrichs, Ruppert 1978; insects, Parker 1970, Thibout 1979; turkeys, King et al. 1999; prosobranch snails, Buckland-Nicks and Chia 1981; aplysiid sea slugs, Thompson 1976). Under our hypothesis, the unified beating of the flagella of resident sperm would generate resistance to incoming sperm entering the tubules; the higher the number of resident sperm, the stronger the resistive force. Thus, the probability of any sperm gaining entrance to the storage organ would decrease with each successive mating, and the advantage enjoyed by the penultimate donor would depend on the number of allosperm stored prior to its mating. A trend in our data, while not statistically significant, provides a level of support for this hypothesis: increasing numbers of offspring attributed to outside fathers corresponded to increasing P_2 values. This hypothesis might also explain why pulmonate sperm are longer than those of all other molluscs (~660μm in *Helix aspersa*; Maxwell 1977), as the beating of longer sperm should generate a greater force.

The ability of sperm from earlier matings to resist incoming sperm might also depend on their condition. If old allosperm provide less resistance than more recent

arrivals, we would expect first-male precedence to decrease with increasing mating interval. Indeed, Baur (1994) found that mean P_2 scores increased from 0.34 when both matings took place during the same breeding season (within 70 days of each other) to 0.76 when the interval between matings exceeded 300 days. We found no evidence of an effect of intermating interval on P_2, but the range of time intervals in our data was extremely limited. However, the small contribution of outside fathers to most of the clutches we examined could be attributed to the reduced competitiveness of allosperm after prolonged storage.

The number of sperm reaching the entrance of the storage organ has importance for both first and second donors beyond the absolute quantity of eggs they can fertilize. Since the first donor's sperm enjoy unrestricted access to the spermatheca, larger numbers provide paternity assurance through increased resistance to the incoming sperm from subsequent donors. If each of the second donor's sperm has an equal probability of overcoming the resistance of the resident sperm, larger numbers reaching the entrance translates into a greater representation of the second male's sperm in the storage organ. The number of sperm reaching the entrance of the spermatheca can be augmented by preventing the recipient from digesting transferred allosperm through dart shooting or by transferring larger ejaculates. Since larger animals produce larger spermatophores (Rogers and Chase 2001), we would expect donor shell volume to influence P_2 scores. Indeed, our results indicate a marginally significant bias in paternity favoring the larger donor in each mating triad.

The described mechanism of sperm utilization in helicid snails remains to be conclusively demonstrated and we propose it only as the best current explanation of the observed paternity distribution. Moreover, it is impossible to definitively state that

manipulation of the FPSC did not stimulate activity in sperm that are normally quiescent in the storage organs. However, we feel that all available evidence supports this mechanism. While largely neglected in the literature, the activity of sperm in storage organs may have consequences for the study of sperm competition in a wide range of animals.

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We thank M.A. Landolfa and A. Davison for instruction in molecular techniques and D.M. Green for the use of his equipment and facilities, as well as G.M. Ntim for snail care. This work was supported by a NSERC Canada research grant to RC and a NSERC Canada PGS-A fellowship to DWR. The experiments described above comply with all current Canadian laws.

Table 1. Selected generalized linear model for factors affecting the proportion of offspring fathered by the second sperm donor (P_2) in twice-mated snails (n = 35). The term "shooting order x maternal volume" indicates a statistical interaction. The term "outside fathered" indicates the proportion of offspring in a clutch fathered by donors from matings preceding those observed. A constant was included in this model since the regression line does not pass through the origin.

Factor	*Coeff*	*df*	*MS*	*F*	*P*
Dart shooting order	-0.786	1	0.899	19.28	<0.001
Shooting order x maternal volume	0.154	1	0.650	13.93	0.001
Donor volume difference	-0.066	1	0.193	4.13	0.051
Outside fathered	0.455	1	0.148	3.18	0.085
Error		30	0.047		

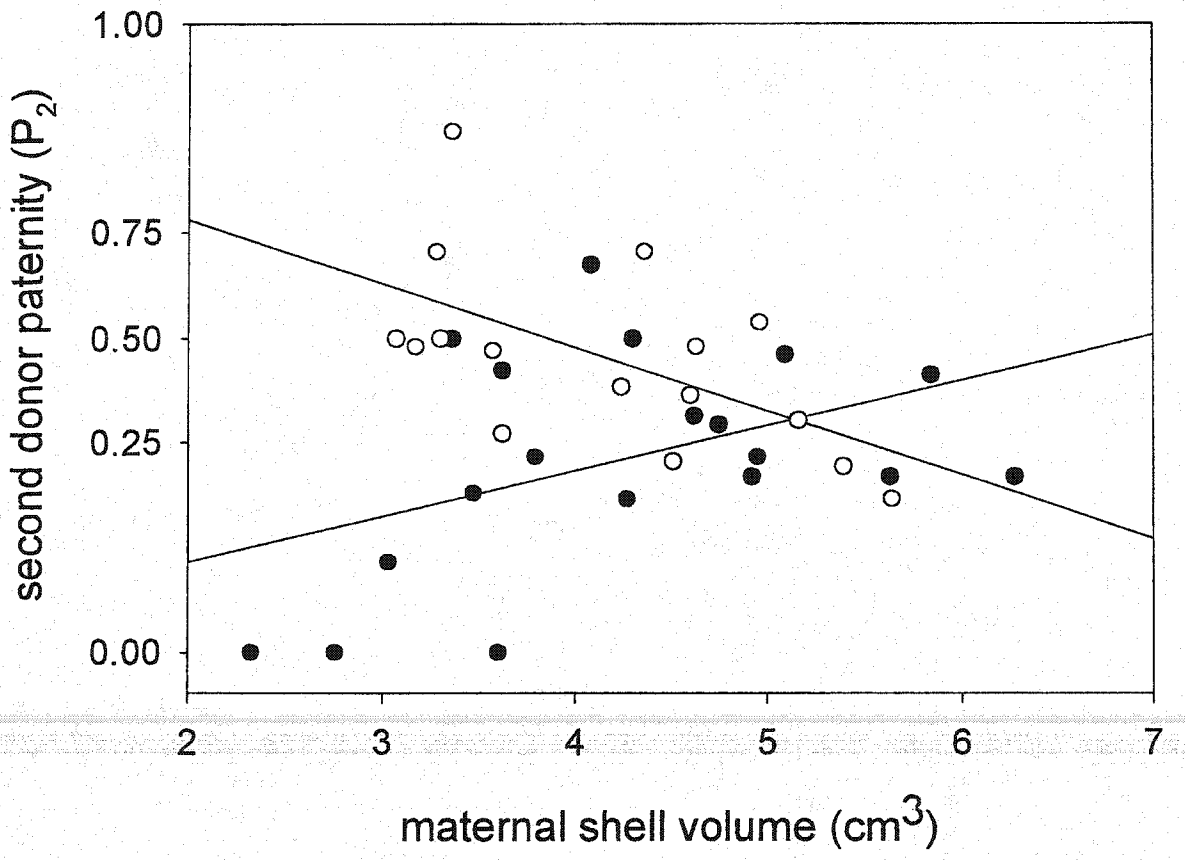
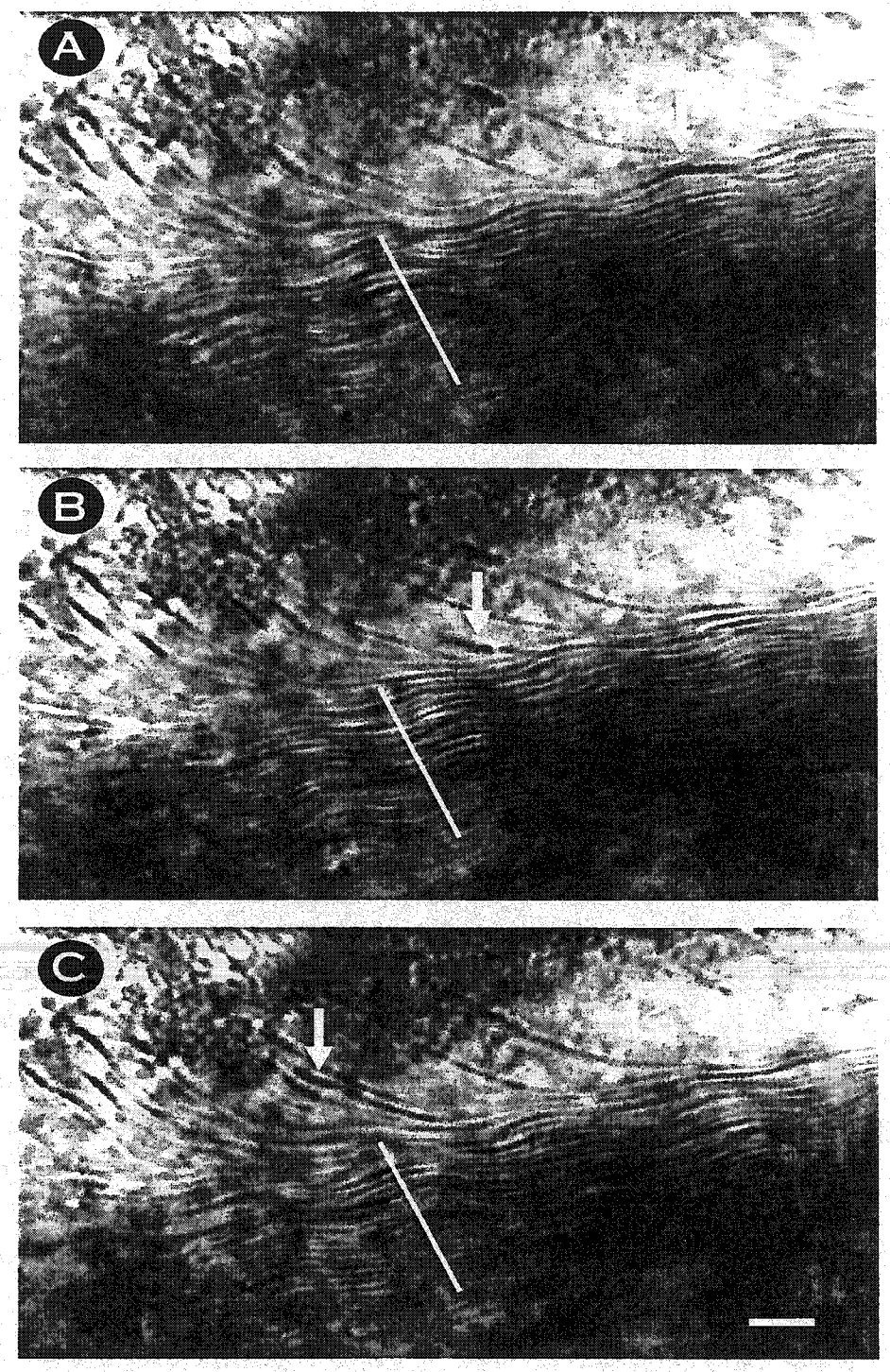


Fig. 1 Effect of dart shooting order and maternal shell volume on the proportion of offspring fathered by the second sperm donor (P_2). Regression lines indicate the dependence of P_2 on maternal shell volume for each dart receipt category (*closed circles* only first donor's shot hit recipient, *open circles* only second donor's shot hit recipient).

[Figure on next page]

Fig. 2 Activity of allosperm within the spermatheca. Three still frames captured from a video of a storage tubule (near the blind end) at t = 0.0 sec (A), t = 4.3 sec (B), and t = 8.5 sec (C). Note the progress of the sperm head indicated by the white arrow as it moves through the beating tails in the lumen against the direction of wave propagation (A-B) and into the spermathecal epithelium (C). The solid white line is located in the same position in each frame and provides a reference for observing the synchronous beating of the tails. In A, the line is situated on a maximum of the sinusoidal wave formed by the beating tails. In B, the line in near the minimum and in C it is once again closer to the maximum. The frequency of tail beating is approximately 0.7 sec^{-1}. Scale bar = 12 μm.


A
B
C

Chapter 4

GENERAL DISCUSSION

The primary goal of this thesis is to explain the adaptive significance of dart shooting. I have tested two predictions derived from the sperm loading hypothesis. In Chapter 2, I present evidence that snails struck by darts store significantly more sperm than do snails that are missed. Furthermore, in Chapter 3, I demonstrate that this biased storage translates into higher paternity for successful shooters in the clutches of multiply mated recipients. These findings provide an answer to three centuries of questions about the function of the dart, and suggest an important role for postcopulatory sexual selection in snails and other simultaneous hermaphrodites. However, the results included in these two chapters are not limited to the effect of dart shooting. In Chapter 2, I demonstrate the importance of body size to sperm competition in snails. Donor shell volume influences the number of sperm transferred while recipient shell volume influences the number of sperm stored and the efficacy of dart receipt. Furthermore, in Chapter 3, I provide evidence of first donor sperm precedence and I propose a novel, albeit speculative, mechanism of sperm utilization in *H. aspersa*. This mechanism, while requiring further investigation, could have important consequences for the study of sperm competition in many taxa.

Although I have explained the adaptive significance of dart shooting in *H. aspersa*, many questions remained unanswered. In this section, although I will elaborate on some of the points made above, I shall not revisit all of the results presented in this thesis as they have been discussed at length in their respective chapters. Instead, I will address some of the problems with our current understanding of dart shooting and other aspects of courtship, copulation and sperm utilization in snails, and suggest avenues for future research.

The proximate mechanism

From the evidence described above we can conclude that dart shooting influences sperm storage and paternity in *H. aspersa*. As discussed in Chapter 2, the strength of this conclusion is limited by the correlational nature of the demonstrated association between dart receipt and reproductive success. It remains possible that undescribed factors linked with dart shooting success are responsible for the observed results. However, the demonstration of a direct physiological effect of digitiform mucus on the recipient (Koene and Chase 1998b) provides strong experimental evidence of a causal relationship between dart receipt and biased sperm storage and paternity.

Although Koene and Chase (1998b) clearly demonstrated a physiological response the female tract of the recipient, they could only speculate about the consequences of this response. Consequently, the proximate mechanism underlying the effect of the dart requires further elucidation. The authors described a twofold response of the isolated female reproductive tract to *in vitro* application of a homogenate of the digitiform glands. First, homogenate application increased the frequency of peristaltic contractions in the bursa tract diverticulum for a period of 30-60 min. Second, homogenate application caused conformational changes in the copulatory canal which, the authors speculated, closed off the entrance to bursa tract (which leads to the gametolytic bursa copulatrix). This conclusion was based on visual observation of the copulatory canal and the use of a spermatophore-shaped probe. They report that prior to homogenate application, the probe could enter the bursa tract but not the diverticulum. The situation was reversed after application of the homogenate.

Koene and Chase (1998b) suggested that the increased frequency of peristaltic contraction in the bursa tract diverticulum speeds the uptake of the spermatophore, which is reasonable, but they then proposed that faster uptake will allow more sperm to escape digestion. Since the mechanism and timing of sperm activation are poorly understood, the relationship between the speed of spermatophore uptake and sperm survival is currently unknown. It is possible that sperm do not become active until spermatophore transfer is complete, in which case the speed of uptake would be irrelevant. Moreover, if sperm activation begins at the initiation of transfer, faster uptake might actually reduce sperm survival as they would be subject to digestion for a longer period of time. Indeed, Koene and Chase (1998a) initially predicted that the digitiform mucus should slow the rate of contraction in the diverticulum. Finally, increased peristalsis was only observed for a maximum of sixty minutes (the duration of trials) but the spermatophore is not transferred until at least four hours after the initiation of copulation (Adamo and Chase 1988). Consequently, the rate of peristalsis may return to normal during the three hour interval between the end of the observations and the initiation of spermatophore transfer.

The induction of conformational changes in the copulatory canal is more relevant to the proposed mechanism of dart function. The observation that a spermatophore-shaped probe could enter the bursa tract prior to, but not after, application of the homogenate seems convincing. However, in *H. aspersa*, the spermatophore is always received into the diverticulum, not the bursa tract, regardless of the success of dart shooting (Adamo and Chase 1988). Instead, sperm escaping from the tail of the spermatophore are pulled up into the bursa copulatrix through peristaltic contraction in the bursa tract. The fact that an object as large as a spermatophore did not enter the bursa tract after homogenate application does not imply that access to sperm will also be

restricted. Finally, many dart shooting species have extremely reduced or altogether absent diverticula (e.g. *Helix pomatia*, Meisenheimer 1912). In these animals the spermatophore is always transferred directly into the bursa tract. Clearly, the mechanism proposed by Koene and Chase (1998b) cannot be extended to these species.

Koene and Chase's (1998b) mechanism remains a valid hypothesis for *H. aspersa* but their observations support several alternative hypotheses. For instance, the digitiform mucus might simply facilitate spermatophore transfer. In many cases, the spermatophore tail is not completely internalized by the recipient for several hours after the end of copulation. If the spermatophore is not completely internalized, sperm might follow the tail out of the recipient's reproductive tract where they would be wasted. The probability of complete spermatophore transfer during copulation is increased fivefold when the recipient is struck by a dart (Koene and Chase 1998b), likely due to the increased rate of peristalsis in the female tract associated with dart receipt. This mechanism would be particularly advantageous in species like *H. pomatia* where copulating animals withdraw their penes shortly after the initiation of transfer and remain motionless for up to 9 hours while internalizing the spermatophore tails (Lind 1976). During this time, part of the spermatophore is exposed to external conditions. Faster and more reliable uptake would increase the probability that the spermatophore is internalized without damage, thereby promoting sperm survival.

Runaway sexual selection

Adamo and Chase (1996) labeled the dart an 'instrument of male manipulation', suggesting that dart shooting is an attempt by the male (shooter) to usurp the female's

(recipient's) control over fertilization and force her to use his sperm. Koene (1999) elaborated on this hypothesis and created a false dichotomy, suggesting that manipulation and female choice were mutually exclusive hypotheses. However, his extreme interpretation of manipulation is based on the assumption that preferentially using the sperm of successful shooters is not in the interest of the female. This assumption is logically untenable.

Specialized organs for the digestion of allosperm exist in all gastropod groups. Indeed, female tract hostility is a universal feature of internal fertilizers (Birkhead et al. 1993). Any male trait, such as the dart, which reduces this hostility and promotes allosperm survival will likely increase male reproductive success and generate a selective advantage for males possessing the trait. These adaptations might be particularly common in hermaphrodites, since the factors controlling the female reproductive tract are readily accessible to the male component (Adamo and Chase 1996). Thus, the biased utilization of allosperm associated with dart receipt likely arose through exploitation (i.e. manipulation) of a pre-existing motor system in the female tract. No important distinction should be drawn between the transfer of substances that bypass sensory systems and induce direct physiological responses (allohormones; Koene and ter Maat 2001) and traditional signals whose effects are mediated through external sensory organs. The former mechanism of exploitation is no more manipulative than the latter. That is, the dart is comparable to acoustic signals like the 'chuck' of male tungara frogs (Ryan et al. 1990) or visual signals like the long tails of swordtail fish (Basolo 1990) that exploit pre-existing female biases.

Once the ability of the dart to manipulate the female reproductive tract is established, dart shooting could be subject to a runaway process (Fisher 1958; Charnov

1979). Females benefit from using the sperm of better shooters because, assuming shooting success is heritable, offspring sired by good shooters will enjoy the same reproductive advantage as their fathers. Under runaway selection, males evolve stronger signals and females evolve preferences for increasingly strong signals. At some point the cost of producing large signals typically balances the reproductive advantage gained through signaling and the runaway process reaches an end point.

The hypothesis that dart shooting exploits a pre-existing female motor bias could be tested by examining the effects of the digitiform mucus of one species on a closely related species that never evolved darts. We would expect the mucus to have strong effects on sperm digestion in the dartless species.

Female resistance

In most systems, the cost of elaborate signals is borne solely by the male. However, it is easy to imagine that larger darts or more concentrated mucus might harm the recipient more than the shooter, possibly through increased trauma associated with receipt or increased toxicity of the mucus. My own attempts at mucus injection resulted in death of the recipients (data not included), suggesting that the digitiform mucus is toxic; this hypothesis requires further investigation. Under such circumstances selection might drive females to evolve resistance to, rather than preference for, the dart. Holland and Rice (1998) proposed that the evolution of female resistance might result in higher stimulatory thresholds (the minimum amount of mucus required to close off the gametolytic gland). For example, if the digitiform mucus is diluted in the reproductive tracts of larger recipients (Chapter 2), increased body size might reduce the costs of dart

receipt (through reduced mucus toxicity and relatively smaller puncture wounds). In response, selection would drive the evolution of more extreme male traits resulting in cyclic antagonistic coevolution (chase-away selection, Holland and Rice 1998). To continue the body size example, since dart efficacy is reduced in larger recipients (Chapter 2), selection for increased body size should counter-select for larger darts or more concentrated mucus. This process would ultimately be limited by the extent to which the fitness cost suffered by the recipient affects the reproductive success of the shooter. If dart receipt results in a large decrease in the fecundity of the recipient (possibly by causing death), the shooter's own reproductive success will suffer. Once dart shooting has obtained its maximum effectiveness, constrained by both production costs and decreased fecundity of the recipient, female resistance may continue to increase to the point where the dart is rendered completely ineffective.

Interspecific comparisons reveal considerable diversity in dart shooting behavior. Certain species, like *H. aspersa*, are obligate shooters (Adamo and Chase 1988). Others, including *A. arbustorum*, shoot only occasionally (Baminger et al. 2000). Still others, including several helminthoglyptids, have completely lost their dart apparatus (Roth 1996). While the reduced importance of dart shooting in certain species might be attributable to high levels of female resistance, other explanations are possible. High costs associated with dart shooting, either through producing the dart or through reducing the fecundity of the recipient, should prevent indiscriminate shooting. Indeed, costly darts should only be shot when the potential returns are highest, such as under conditions of high sperm competition risk. Certain non-adaptive mechanisms, such as genetic drift and the temporary relaxation of selection during colonization, could also explain the reduced importance of the dart in some species.

The diversity of darts

Not only do interspecific comparisons reveal differences in dart shooting behavior, they also expose a remarkable diversity in dart structure. If the sole function of the dart is to transfer mucus through the body wall of the recipient, one might expect to find a single optimal structure common to all species. The interspecific diversity of dart structure exists in stark contrast to the extremely limited intraspecific variation (Tompa 1984). This might be an artifact of using dart structure as a systematic character. Species-level distinctions in gastropod systematics are not based on known patterns of reproductive isolation (the biological species concept, Mayr 1944) but rather on morphological differences (although molecular evidence is gaining importance, see Wade et al. 2001). Since morphology-based phylogenies are often poorly resolved at the species level, species might be distinguished on the basis of differences in a small number of characters, such as dart morphology, that exhibit high levels of variation between closely related groups. Consequently, if populations are sometimes designated as different species on the basis of dart structure we would expect high levels of variation between, but not within, species. For example, while dart morphology is used to distinguish *Cepaea nemoralis* from *Cepaea hortensis* (Tompa 1980), these two species are capable of hybridizing (Lang 1908). As a result, the variation in dart structure among reproductively compatible groups of snails is likely greater than currently estimated.

Speciation among gastropods is poorly understood. It seems likely, given the leptokurtic dispersal patterns exhibited by land snails (Davison and Clarke 2000), that new species arise through a small number of long distance migrants (potentially as few as one) becoming reproductively isolated in novel habitats. If this assumption is true, then

interspecific differences in dart structure might simply be a non-adaptive consequence of the founder effect. During the early stages of colonization, the combined influence of non-representative sampling of migrants, high levels of genetic drift (including inbreeding) and a temporary relaxation of selection might cause dart structure in the new population to diverge from that of the parent population. Indeed, the relaxation of selection during the flush phase of colonization allows recombinant genotypes (normally removed by natural selection) to survive causing the breakup of coadaptations and supergenes (Carson 1975). This genetic disorganization can result in novel genotypes (Carson and Wisotzkey 1989). Moreover, high levels of inbreeding can generate homozygous allele combinations in the new population that are exceedingly rare in the parent population. Once the new population has become established, selection to maximize the mucus-transferring capacity of the dart will be constrained by the existing variation resulting in a new optimal dart structure.

Honest signaling

As an alternative to runaway selection hypothesis, the dart may serve as an indicator of the innate quality of the shooter (i.e. an honest signal). If some aspect of dart shooting is correlated with the viability of the shooter, and viability is heritable, then females would benefit from using the sperm of better shooters as the resulting offspring would have greater viability than those fathered by poor shooters (Landolfa in press). Since the environment faced by snails is largely unpredictable, the viability component indicated by dart shooting must be advantageous across a wide range of environmental conditions. At this time, we can resurrect the calcium hypothesis. Charnov (1979)

suggested that the dart might indicate the shooter's ability to acquire and metabolize calcium, a trait important for the growth and development of offspring in any environment (the resource-accrual hypothesis; Trivers 1976). Indeed, young snails better able to acquire and metabolize calcium should be more viable than those that do so poorly. Koene and Chase (1998a) found that depriving snails of calcium for eight months had no effect on their ability to produce darts. However, this observation might simply underline the importance of dart shooting as snails may have been mobilizing calcium from their shells (Tompa and Wilbur 1977) to produce darts. Indeed, Koene and Chase (1998a) found that calcium deprived snails had exceptionally thin shells.

If dart shooting ability is correlated with some aspect of shooter viability, or if dart receipt imposes a large cost on the recipient, it would be adaptive for snails to attempt to avoid their partner's shots. Avoidance of poor shots could allow recipients to preferentially use the sperm of higher quality shooters or reduce the costs of mating. This establishes a conflict during courtship, where shooters attempt to strike recipients and recipients attempt to avoid their partners' shots.

Courtship as conflict

While the function of dart shooting has been resolved, other elements of the protracted and elaborate courtship ritual of *H. aspersa* remain unexplained. Dart shooting is likely the most important element of courtship, and the other behaviors (such as biting and lip-genital contact) can be interpreted in this light. Giusti and Lepri (1980) suggested that biting during courtship might be used to test the sexual interest of a mating partner. Koene (1999) modified this hypothesis with respect to dart shooting. Since withdrawal

responses are suppressed during mating (Balaban and Chase 1990), Koene (1999) suggested that snails bite their partners in order to test their avoidance behavior. That is, if the partner does not withdraw in response to a bite, it will likely not be able to avoid the shot dart. This hypothesis is supported by the fact that snails generally do not bite after shooting their darts.

The extended length of courtship can be attributed to snails attempting to avoid their partners' shots. In order to shoot, a snail must tightly appose its genital atrium against the body wall of the recipient and adopt a rigid posture (Adamo and Chase 1988). When preparing to shoot, they are vulnerable to being darted by their partners. Consequently, snails should be hesitant to shoot first, particularly since the shooter becomes less cautious after its shot (probably in an attempt to ensure that its own dart is not wasted) making it an easier target. Once the first snail shoots, the second need no longer fear being hit and can shoot its own dart with impunity. However, the second snail will likely only know that its partner has shot if it is struck by the dart. This hypothesis explains Adamo and Chase's (1988) observation that the interval between the first and second shot is significantly shorter when the first shot hits, rather than misses, the recipient.

Thus, helicid courtship can be viewed as a conflict between two armed opponents. Each animal repeatedly tests the other, attempting to determine when the opponent might strike, or when it is most vulnerable to being shot. Snails might even use lip-genital contact (where one snail rubs its partner's genital atrium with its buccal apparatus; Adamo and Chase 1988) to cause their partners to shoot prematurely. These conflict-based hypotheses for lip-genital contact, biting, and the duration of courtship can be tested by comparing the courtship rituals of dart-bearing and dart-lacking species.

Sperm utilization

The utilization of sperm from different donors, biased by dart receipt, is also influenced by the order or mating. The evidence for first donor sperm precedence described in Chapter 3 is interesting for two reasons. First, no previous study has conclusively demonstrated any effect of mating order on paternity in molluscs. Second, first donor precedence is rare across all taxa with the exception of the arachnids (Elgar 1998). Since existing hypotheses for first donor precedence fail to explain its occurrence in snails (reviewed in Chapter 3), I have proposed a novel mechanism based on the motility of stored allosperm. It should be noted that I invoke this mechanism only as a possibility. I cannot definitively state that the observed activity is not an artifact of experimental manipulation of the sperm storage organs. However, the same level of skepticism should be applied to reports of immotility.

Sperm in the storage tubules of birds are widely believed to be quiescent. Indeed, numerous studies have described physiological adaptations presumed to maintain stored sperm in an inactive state (reviewed by Bakst et al. 1994). However, the only evidence that stored sperm are inactive stems from unpublished observations of squash preparations (cited in Bakst 1987). While the pressure applied to the sperm storage tubules in these preparations could very well constrain the movement of sperm, Bakst actually observed undulating sperm in some of the squashed tubules. Moreover, using a mechanism to isolate storage tubules without squashing, King et al. (1999) reported that the stored sperm beat in a slow synchronous motion (exactly as described in Chapter 3). Despite the inconclusive evidence, researchers studying most organisms presume that sperm are stored in a quiescent state.

The motile sperm storage hypothesis provides proximate mechanisms for two poorly understood phenomena: passive sperm loss and the coevolution of the lengths of sperm and sperm storage tubules. Second male precedence in birds in generally attributed to passive sperm loss (Birkhead, 1998). Following insemination, sperm slowly leak out of the storage tubules. Thus, the number of sperm stored from each donor decreases over time, resulting in biased paternity favoring the last male to mate prior to oviposition. The mechanism underlying passive sperm loss is unknown. The orientation of sperm in the storage tubules of birds is identical to the orientation in snails; the heads are generally located at the blind ends with the tails extending out towards the opening (Bakst et al 1994). If the stored sperm beat synchronously (as observed by King et al. 1999) they will generate a force vector directed out of the tubule. Any sperm that stops beating, or merely slows down, will be pushed out of the tubule resulting in the observed patterns of passive sperm loss. Variation in the duration of sperm motility could be attributed to differences in energy reserves or haploid gene expression between sperm.

Sperm length appears to be an important factor in sperm competition. Indeed, sperm length is correlated with the intensity of sperm competition in passerine birds (Briskie et al. 1997) and shorebirds (Johnson and Briskie 1999). However, the source of the competitive advantage enjoyed by long sperm remains unexplained. Longer sperm are expected to generate greater propulsive force (Katz et al. 1989), but this advantage is offset by increased drag (Wu 1977). Indeed, sperm length is not correlated with swimming velocity in passerine birds (Birkhead 1998). Moreover, sperm length is not correlated with the thickness of the zona pellucida in mammals (Gomendio and Roldan 1993), suggesting that higher propulsive forces do not help long sperm penetrate the ova vestments.

Interestingly, sperm length coevolves with the length of the sperm storage organs in both birds (Briskie et al. 1997) and insects (featherwing beetles, Dybas and Dybas 1981; stalk-eyed flies, Presgraves et al. 1999; fruit flies, Pitnick et al. 1999). Briskie and Montgomerie (1992) argued that long sperm evolved to fill the spermathecal tubules, preventing the sperm of subsequent males from entering the storage organ (spermathecal filling; see Chapter 3). Longer tubules evolved in response, thus restoring a measure of control over fertilization to the female and perpetuating the coevolutinary cycle. However, space in the sperm-storage tubules is rarely limiting, particularly in birds (Bakst et al. 1994). Consequently, long sperm are unlikely to provide paternity assurance simply through occupying more of the available storage space. As suggested in Chapter 3, the force created by the synchronous beating of resident sperm tails should generate resistance to incoming sperm even when the storage tubule is not full. Longer sperm will generate stronger resistive forces. We can ignore the associated higher drag since stored sperm do not exhibit forward displacement. Moreover, long sperm might be able to displace shorter resident sperm from the storage tubules. Thus, under the motile sperm storage hypothesis, long sperm might provide males with competitive advantages in terms of both the offensive and defensive abilities of their sperm.

Although land snail sperm have large glycogen reserves (Maxwell 1983), prolonged maintenance of activity in storage likely requires nutritional provisioning by the recipient. Several studies have attributed a secretory function to the spermathecal epithelium (Giusti and Selmi 1985, Bojat et al. 2001). The nature of these secretions is unknown, but glycogen remains a viable candidate. Presumably, maintaining active sperm in storage would exact a metabolic cost on the recipient. Indeed, the cost of storing sperm might explain why recipient snails (or female gonochorists) digest or expel the vast

majority of sperm received. Why then would snails nourish allosperm? One possible answer is that sperm activity must be maintained in order for the sperm to remain capable of fertilization; the metabolic expense would be the price paid for uncoupling copulation and oviposition. However, it is possible that the recipient benefits from storing motile sperm. For instance, it could provide a basis for sperm selection throughout the storage period as old, diseased or low quality sperm would be pushed out of the tubules by more viable sperm.

New techniques may allow us to test the motile sperm storage hypothesis. The miniaturization of endoscopic techniques might soon offer a relatively non-invasive way of viewing sperm within the storage organ. Until this time, the quiescence of stored allosperm should not be presumed.

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