Reproduction in the Hermaphrodite
_Aeolidiella glauca_

_A Tale of Two Sexes_

BY

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Abstract


This thesis focuses on reproduction in a simultaneous hermaphrodite with internal fertilization; the nudibranch *Aeolidiella glauca*. Unlike most other nudibranchs, where copulation is the rule, *A. glauca* was found to transfer sperm via external spermatophores that were attached to the partner’s back. Despite elaborate courtship the actual spermatophore transfer, which always involved two animals only, was of short duration. In most matings (88%) spermatophores were reciprocally exchanged. *A. glauca* was further found to be very promiscuous. During mating and sperm transfer the receiver exerts considerable control over sperm, and manipulative behaviours designed to increase the donor’s reproductive success are thus likely to have evolved. An example of such manipulative behaviour may be *A. glauca*’s unique spermatophore avoidance behaviour. I found that slugs carrying a sign of previous mating activity, i.e. a spermatophore, were discriminated against in a situation where mate choice was possible. Furthermore presence of a spermatophore was found to reduce the carrier’s ability to successfully interrupt matings between other slugs. Body size, however, had no direct effect on displacement in *A. glauca* as small slugs interrupted matings as successfully as large ones. Furthermore, pair formation and mating were found to be random with respect to size. This was true also for pairs formed in the field.

In addition to mate choice hermaphrodites may increase their reproductive success by differential sex allocation. I tested whether differing mate encounter rates had any effects on allocation to male and female function in *A. glauca*. Slugs with more mating opportunities mated more, and had higher proportional spermatophore production that others. As predicted they also laid significantly fewer eggs than slugs presented with partners less often.

*Keywords*: *Aeolidiella glauca*, simultaneous hermaphrodite, mating behaviour, mate choice, sex allocation.

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“Result! Why, man, I have gotten a lot of result. I know several thousand things that won’t work”

- Thomas A. Edison

“It was the best of times, it was the worst of times…”

- Charles Dickens, “A Tale of Two Cities”
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-V.


II. Karlsson, A. & Haase, M. The enigmatic mating behaviour of a simultaneous hermaphrodite, the nudibranch *Aeolidiella glauca* (Gastropoda, Opisthobranchia). Submitted manuscript.


V. Karlsson, A. Sex allocation in a naked hermaphrodite. Submitted manuscript.

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The order of the authors reflects their involvement in the papers. I have personally written and performed all analyses of papers II, III, IV and V. My co-author in papers II and III collected data, discussed ideas, analyses and commented on the text. In paper I I collected part of the field data, discussed ideas and commented on the text.
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INTRODUCTION

This thesis deals with different aspects of reproduction in a simultaneous hermaphrodite, the nudibranch *Aeolidiella glauca*. At present, comparatively little is known of mating systems of hermaphroditic animals. However, their reproductive structures often show amazing complexity, and mating behaviours can be very elaborate (Hyman 1951; Lind 1976; Vreys & Michiels 1997; Michiels 1998 and refs. therein). Thus, simultaneous hermaphrodites represent an almost untapped potential for behavioural studies that can increase our understanding of the evolution and maintenance of mating systems.

The aim of this introductory part is threefold; I want to provide the reader with some background information on hermaphroditism and nudibranchs. In addition I wish to give a brief outline of the theory underlying my studies. Finally, I hope to be able to convey some of the excitement and joy that the work on this peculiar and fascinating animal has given me. There’s a whole world of hermaphrodites out there, just waiting to be explored…

What is an hermaphrodite?

An hermaphrodite is defined as an organism possessing both male and female reproductive organs. Hermaphroditism has been known since antiquity, but its functional significance is, as yet, only partly understood. The word hermaphrodite is derived from Greek, referring to the androgynous diety Hermaphroditos. This beautiful bisexual being was the result of an extramarital affair between Hermes and the love Goddess Aphrodite.

In biology hermaphrodites are specifically defined as organisms that possess a functional male and female reproductive system during at least part of their lives. This type of gender expression is widespread throughout the animal kingdom, including entire taxa such as flatworms, pulmonate gastropods and ascidians (Ghiselin 1969). Two-thirds of all phyla contain hermaphroditic representatives, and approximately 40% of the molluscan genera are hermaphroditic (Heller 1993).
Usually two major types of hermaphroditism are distinguished. Sex-changers, or sequential hermaphrodites, start out as one sex and change into the other later in life. Sequential hermaphroditism is relatively well studied, and much research has been devoted to the question of when to change sex (Warner 1975, 1988; Berglund 1986, 1991; Charnov 1982). The second type, and the focus of this thesis, are simultaneous hermaphrodites. These have functional male and female genitalia simultaneously present for most of their lives, and reproductive acts usually involve both the male and female function in each individual.

Nudibranchs

The order Nudibranchia – the name meaning "with naked gills – is the major representative of the molluscan subclass Opisthobranchia. All nudibranchs are marine, and in the adult stage both shell and operculum have been lost. The loss of shell has allowed for a diverse array of body forms, and not suprisingly nudibranchs are often referred to as “sea-slugs”. Following the classification outlined by Thompson (1976), nudibranchs are divided into four suborders; Dendronotacea, Doridacea, Arminacea and Aeolidacea (Fig. 1).

Since nudibranchs lack a shell they may appear more vulnerable to predators than other gastropods. However, to compensate for the loss of a hard shell nudibranchs have developed a wide range of defensive mechanisms, mainly through prey exploitation. Most nudibranchs are browsing predators, and they often have very limited and specific food preferences. Dorid nudibranchs (for examples see Fig. 1) mainly feed on sponges and bryozoans, and many have the ability to synthesize unpleasant or poisonous chemicals, usually as secondary metabolites from the prey. Aeolids prey on cnidarians, and have evolved an immunity to the damaging effects of nematocysts. Interestingly, they can also use the nematocysts for their own defence. The nematocysts are transported undigested to the apices of the digestive gland in the cerata, and if disturbed the nudibranch can release a whole cloud of discharging nematocysts in the direction of the potential danger. To date, the mechanisms behind this intriguing use of nematocysts are, however, completely unknown.

Their frequently exotic colouration and appearance have made nudibranchs highly popular among SCUBA-divers and naturalists, while other properties such as their ability to synthesise chemical compounds for anti-predatory defence, have made them interesting for bio-medical research and other branches in the scientific community. But despite their wide popularity, comparatively little is actually known about nudibranchs.

**Reproductive anatomy of nudibranchs**

All nudibranchs are simultaneous hermaphrodites with internal fertilization. To date there is no evidence suggesting that self-fertilization takes place among nudibranchs. Their reproductive systems are often complex (Ghiselin 1966; Hyman 1967; Schmekel 1971, Hadfield & Switzer-Dunlap 1984, Gosliner 1994), but most species copulate and exchange sperm reciprocally. A few species, however, transfer sperm via hypodermic impregnation (Rivest 1984), or in a spermatophore (von Ihering 1886). Sperm, even when transferred in a spermatophore, is usually confined within the reproductive system.

Nudibranchs have a single unpaired gonad, the ovotestis, which is composed of
individual acini or follicles. It may be difficult to distinguish between male and female parts, but frequently a single male acinus is surrounded by several female. However, eggs and sperm are occasionally produced by the same acinus.

The gametes are transported through the hermaphroditic duct to the ampulla, where autosperm, i.e. own sperm, is stored until copulation takes place. The ampulla opens into the spermoviduct, which connects to the *vas deferens* and, via an oviduct, to the fertilization chamber. Eggs are transported directly to the fertilization chamber. In contrast autosperm is channeled through the *vas deferens*, to the *ductus ejaculatoris* and the evertible penis. The *vas deferens* usually contains a glandular part, the prostrate. The function of the prostatic fluid is not completely clear, but it may help to agglutinate the sperm into a ball (Schmekel 1971). A great diversity is found in the penes of nudibranchs, often including various appendages and spines. Many of these specializations are of taxonomic value.

The distal female genitalia can be organized according to a diaulic or triaulic scheme. Schmekel (1970) distinguished two diaulic types and a single triaulic type among nudibranchs (Fig. 2). All Doridacea are triaulic, whereas most Aeolidacea and Dendronotacea belong to one of the diaulic types.

Nudibranchs with a triaulic reproductive system have separate orifices and ducts for receiving allosperm and for laying the fertilized eggs. A vagina leads to the *receptaculum seminis*, where allosperm, i.e received sperm, is stored. Close to the receptaculum is a copulatory bursa, where allosperm is digested. A duct then leads sperm to the fertilization chamber. Fertilized eggs pass through another duct with a series of glandular parts. Here eggs are provided with the protective layers that form the spawn mass. The morphology of the glands is extremely complex, and the terminology thus confused. In general three glands are recognized, which are named according to function. The albumen gland adds nourishment to the eggs whereas the membrane gland secretes a thin egg membrane. The mucus gland finally, secretes the gelatinous layers that hold the eggs together, and give the completed spawn its specific shape.
Diaulic nudibranchs lack the copulatory bursa, and have a common orifice and duct for sperm reception and egg laying. The principal difference between the diaulic types is where the spermoviduct branches into oviduct and *vas deferens*.

**Fig. 2.** Schematic diagrams of the three major patterns of reproductive anatomy found in the Nudibranchia. **A.** Diauly I. **B.** Diauly II. **C.** Triauly. Abbreviations: Am, ampulla; Cb, copulatory bursa; Ed, ejaculatory duct; Me, membrande gland; Mu, mucus gland; Ot, ovotestis; Ov, oviduct; Pe, penis; Poa, postampullar duct; Pr, prostate; Pra, preampullar duct; Sr, seminal receptacle; Va, vagina; Vc, vaginal duct; Vd, Vas deferens; Ve, vestibule. (After Schmekel, 1971)

**Hermaphrodite reproduction**

Genital morphology plays a central role in the systematics of many animal groups. It has been proposed that a principal factor contributing to the vast diversity of genitalia is sexual selection (Eberhard 1996). In order to understand how sexual selection processes operate in a certain organism it is essential to know the function of its genital system. Among molluscs opisthobranchs are known for their particularly complex and diverse genitalia. Literature
abounds with descriptions of opisthobranch genital systems (e.g. Ghiselin 1966; Schmekel 1970, 1971), but nevertheless detailed functional analyses are comparatively scarce, and organs are often named only according to their arrangement, rather than function.

Many hermaphrodites display an amazing array of mating behaviours (Baur 1998; Michiels 1998), but unfortunately reports of these behaviours are often reduced to anecdotal statements. This is particularly true for simultaneous hermaphrodites with internal fertilization. Thorough empirical investigations, such as those on the nematod Caenorhabditis elegans (Wood 1988), flatworms (Hyman 1951; Kearn & Whittington 1992; Peters et al 1996; Vreys et al. 1997a, b) or pulmonate gastropods (see Baur 1998), have provided important steps towards the understanding of the reproductive mechanisms connected with hermaphroditism. However, our present knowledge still covers only a small section of behavioural diversity within hermaphroditic taxa (cf. Ghiselin 1969).

My initial observations of the nudibranch Aeolidiella glauca suggested that the only available description of its genital system, given by Tardy (1969), is partly incorrect. Therefore, it was necessary to perform a detailed anatomical investigation of A. glauca’s genital system. This is described and analysed in paper I, including a short account of A. glauca’s unique mating behaviour. Paper II is an extended analysis of A. glauca’s mating behaviour, with new findings on sperm transfer and reception, and details on egg production.

**Sexual selection in hermaphrodites**

In recent years much progress has been made in understanding how the reproductive biology of organisms with separate sexes has been shaped by sexual selection (Andersson 1994; Andersson & Iwasa 1996). In sharp contrast our understanding of sexual selection in simultaneous hermaphrodites (Charnov 1979, Michiels 1998; Greeff & Michiels 1999a, b), where only few model systems exist, remains poor. The absence of hermaphrodites from sexual selection literature cannot be attributed to a lack of a conceptual basis, as many
theoretical contributions have been made over the past decades (Charnov 1979, 1982, 1996; Morgan 1994; Michiels 1998; Greeff & Michiels 1999a).

Although sexual selection should be expected also in hermaphrodites it may be more difficult to identify sexually selected traits in such organisms (Bateman 1948; Queller 1983). Furthermore, certain areas of sexual selection are probably excluded in hermaphrodites (Morgan 1994). For instance, selection on traits related to mate acquisition are likely to be intrinsically weaker in hermaphrodites (Greeff & Michiels 1999a). Instead sexual selection may act more subtly through sperm competition and/or cryptic female choice (Eberhard 1996; Baur 1998).

Hermaphrodite reproductive structures often show a high degree of complexity, and mating behaviours can be very elaborate (Hyman 1951; Lind 1976; Vreys & Michiels 1997; Michiels 1998 and refs. therein). Prolonged courtship provides ample opportunity for partner assessment, and may thus set the stage for mate choice in hermaphrodites. It is, however, less obvious which traits might be of importance in hermaphrodite mating decisions, and studies revealing pre-copulatory mate choice in simultaneous hermaphrodites are few (Tomiyama 1996; Yusa 1996; Vreys & Michiels 1997; Michiels & Bakovski 2000).

Commonly, in hermaphrodites, there is a positive correlation between female fecundity and size (Baur 1988; Baur & Raboud 1988; Yusa 1994; De Witt 1996; Tomiyama 1996; Vreys & Michiels 1997; Wedekind et al. 1998; Trouvé et al. 1999; Madec et al. 2000). As copulations are often costly, due to long duration, increasing risk of predation or complicated transfer of spermatophores (Lind 1976), and as many hermaphroditic species occur at high densities a preference for larger partners may be expected, resulting in size-assortative mating (Ridley 1983). So far, size-related mate choice is known only from a few hermaphrodites (Yusa 1996; Vreys & Michiels 1997).

While size may be important, the mating frequency also affects mate choice. Bateman’s principle (1948) states that multiple matings primarily serve the males’ interests. This is valid also for hermaphroditic animals (Charnov 1979), and hermaphrodites are thus expected to
mate readily, i.e. donate sperm, whenever there are opportunities to do so. However, this is only true provided that the male function remains cheap. When population densities are high, and multiple matings common, animals must invest more in sperm in order to outnumber the reproductive output of their rivals. As a consequence allocation to sperm may become as expensive as allocation to eggs (Greeff & Michiels 1999b), and sperm donors may become choosy about to whom they donate sperm.

Given the fact that hermaphrodites frequently live in high density conditions, and that multiple matings are common in many species (e.g. Kutschera & Wirtz 1986; Baur 1988; Peters & Michiels 1996; Peters et al. 1996; Vreys et al. 1997a) it is likely that sperm competition will be as important in hermaphrodites as in any other animal species. Perhaps in response to this, many hermaphrodites have evolved sperm storage organs and/or specialized means of digesting excess sperm (Giese & Pearse 1977; Tompa et al. 1984; Sluys 1989; Michiels & Streng 1998). These may select for sperm quantity or quality, as suggested in the land snail Arianta arbustorum where the recipient may be able to selectively store and digest sperm from different partners (Haase & Baur 1995).

In papers III and IV the potential for mate choice in A. glauca was investigated. I experimentally tested whether body size (III), or presence of spermatophores (IV), was important when making mate choice decisions. I also tested whether any of these characters give a competitive advantage in contests for mates (III).

Sex allocation
Much of the work on hermaphrodite reproduction has focused on sex allocation. Allocation theory predicts that hermaphrodites, unlike organisms with separate sexes, can adjust allocation of resources to each sex function in response to current conditions, thus making reproduction more efficient. This ability to relocate resources also forms the presumed basis for the evolution of hermaphroditism, as proposed by Charnov et al. (1976). This early model predicts that whenever the pay-off for one sexual function decreases with increasing
investment, and it becomes beneficial to relocate resources into the other sex function, hermaphroditism is expected to evolve. This is based on the assumption that the cost of maintaining both sexual functions is not too high. How well individual hermaphrodites are able to regulate the allocation of reproductive resources to male versus female function in response to the number of potential partners is relatively unknown.

Sex allocation and reproductive conflicts of simultaneous hermaphrodites have been extensively studied in hermaphroditic plants (reviewed by Brunet 1992). In animals most empirical support for relative investment in male or female function come from studies on hermaphrodites with external fertilization (Fischer 1981, 1984; Petersen 1991; St Mary 1994; Sella et al. 1997). Work on internally fertilizing species have shown that individuals allocate proportionately more resources to the male function when mating group size increases (Raimondi & Martin 1991; Wedekind et al. 1998; Trouvé et al. 1999).

Recently sex allocation models specifically designed for obligate outbreeders with sperm storage have been developed (Charnov 1996; Greeff & Michiels 1999b). These models include both sperm competition and sperm digestion, and predict that an increased mating rate leads to an increased allocation to the male function. In the final paper (V) of this thesis I experimentally investigated whether *A. glauca* changed its reproductive output via the male and female function in response to differing mating opportunities.

**NATURAL HISTORY OF AEOLIDIELLA GLAUCA**

*Aeolidiella glauca* (ALDER & HANCOCK, 1845) is one of 21 species belonging to the suborder Aeolidacea. It is a sublittoral species common in shallow eelgrass (*Zostera marina*) beds along the coasts of northern Europe. Although commonly reported to occur in low numbers populations at the Swedish west coast often reach high densities. Food habits are variable, but *A. glauca* predominately feeds on Sagartiid sea anemones, especially
Sagartiogeton viduatus. Like other aeolids *A. glauca* harvest the nematocysts of their prey and use these as defensive weapons against potential predators. Adult animals are normally up to 40 mm long (Thompson & Brown 1984), (Fig. 3).

![Aeolidiella glauca dorsal view.](image)

Fig. 3. *Aeolidiella glauca* dorsal view.

The species is annual and reproduction starts around mid-June. Individuals mate multiply with different partners throughout the season, and produce several egg masses. The egg mass is very distinctive, and consists of a threadlike string which is laid in a spiral directly on the eelgrass (Fig. 4). Eggs hatch as pelagic planctotrophic veliger larvae 7-10 days after spawning, depending on water temperature. Post-spawning mortality is usually complete by the end of August.

![Aeolidiella glauca and its spawn on eelgrass.](image)

Fig. 4. *Aeolidiella glauca* and its spawn on eelgrass. The spawn consists of a threadlike string which is laid in a spiral. On average 1 cm egg string contains approx. 1000 eggs. Photo Anders Axelsson.
GENERAL METHODS

The studies were carried out in the summers of 1996 through 2000 at Klubban Biological Station, Fiskebäckskil (58° 15' N, 11° 28' E) at the Swedish west coast. *Aeolidiella glauca* were collected locally from depths of 2-5 m in eelgrass beds (*Zostera marina*) by snorkelling and diving. In the lab, slugs were housed individually in small plastic cups (250 ml) each with its own supply of running sea water, and were fed daily with sea anemones (*Sagartiogeton viduatus*). The plastic cups were cleaned and excess food removed every second day.

All studies were conducted indoors, but under a natural light, salinity and temperature regime. Most observed matings of *A. glauca* took place in petri dishes (I and II), whereas experiments were carried out in 3.6 l aquaria (III and IV) and 250 ml cups (IV and V). In the experiments clean aquaria/plastic cups were used in all trials so that possible chemical cues were not carried from one trial to the next.

In some studies individual egg strings were measured (II and V), and the proportion of fertilized eggs within each clutch assessed (II). The outline of every egg string was copied onto a tracing paper immediately after spawning. The length of the drawn egg string was then measured to the nearest cm with a cartometric wheel. Egg strings are usually of uniform thickness, and it is therefore sufficient to use only length as an indicator of clutch size. The eggs were then left undisturbed in running sea water for 5 to 6 days, at which time the shells of developing veligers become clearly visible. A small part of each egg string was placed on a glass slide and placed under a light microscope. The proportion of eggs that had developed into shelled veligers was then estimated to the nearest 25%.

In all studies body mass was measured for individual slugs, and used as an estimate of body size. Body mass is a more reliable measure than both length and volume, as these soft animals can stretch and contract. Slugs were weighed on an analytical balance, accurate to the nearest 0.01 g, after having been gently blotted on paper towels.

In all studies slugs, except those that were fixed for histology and SEM, were released after experiments were terminated.
Anatomical investigations

For histological (I, II and IV) analysis the animals were either anaesthetized in 7% MgCl₂ and fixed in 10% formaldehyde or 70% ethanol (I), or directly fixed in 70% ethanol (II, IV). Mating and egg laying animals (I) were instantly killed by pouring boiling Bouin’s solution over them, and slugs were then immediately transferred to 70% ethanol to avoid hardening of the glands. Whole specimens (I,II) or the genital system only (I, IV) were embedded in paraplast and sectioned at 6 or 10 µm. Subsequently, the sections were stained either with Heidenhain’s Azan or hematoxylin-eosin.

For SEM analysis (I, II) animals were similarly anaesthetized and fixed in 3% glutardialdehyde in cacodylate buffer (I) or 1% glutardialdehyde, 10% sucrose (II).

The sections for paper I were drawn with a camera lucida and digitized with a customized program. The genital system was reconstructed using the program SURFdriver version 2.5.5 (Moody & Lozanoff 1998).

RESULTS AND DISCUSSION

Aeolidiella glauca’s genital system and peculiar mating behaviour (I and II)

Aeolidiella glauca’s genital system is of the androdiaulic type (Ghiselin 1966), or diaulic type II (Fig. 2) according to Schmekel’s (1971) terminology. A copulatory bursa is lacking as in the vast majority of aeolid nudibranchs (Hadfield & Switzer-Dunlap 1984).

The gonad consists of numerous bundles of acini in which both egg and sperm are produced. The gametes are released through fine, ciliated gonoducts which open into the ampulla, where mature autosperm is stored.

The male genitalia are rather simple and consist of a long, tubular prostate, which winds dorsally on the female gland mass and continues into the penis. The penis is simple and unarmed, but very large when everted. On the ventral side of the penis a line of hook-shaped
lobes is found. The function of these are unknown, but they might simply represent rudiments of an armature of ancestral species. Recent nudibranchs exhibit a variety of accessory penial organs, stylets and hooks (e.g. Edmunds & Kress 1969; Miller 1974; Rudman 1980; Gosliner 1991, 1994).

From the gonad eggs pass through the ampulla and are transported to the fertilization chamber. Here they are fertilized by allosperm released from the receptaculum seminis. The fertilization chamber has two connections with the female gland mass, and one opening into a blind capsule gland. Eggs enter and leave the capsule gland through the same lobe.

After fertilization eggs pass through the capsule gland and the female gland mass which is comprised of five histologically differentiated parts. This is in contrast to the majority of described nudibranch genital systems, where only two or three units are reported (Ghiselin 1966; Edmunds 1970; Schmekel 1970, 1971; Gosliner 1994). The female glands produce the egg coverings, beginning with the egg membrane secreted in the capsule gland and followed by mucus layers added in the mucus glands. The course of the eggs is given in Figure 5.

![Fig. 5. Schematic representation of A. glauca’s genital system. Arrows indicate course of eggs, dashed arrows “wrong courses”. Abbreviations: a, ampulla; cg, capsule gland; dm, distal mucus gland; fc, fertilization chamber; ga, genital atrium; od, oviduct; pma; proximal mucus gland part 1; pmb, proximal mucus gland part 2; pr, prostate; ps, penial sheath; tpa, terminal part 1; tpb, terminal part 2.](image)
*Aeolidiella glauca* was found to exhibit a most “peculiar” mating behaviour (Fig. 6).

The courtship was usually initiated by one slug following a potential partner around (Fig. 6A). This initiating phase was often quite brief, but animals often approached each other repeatedly. If there was response to the following-behaviour, slugs normally oriented themselves into a head-to-head position. When animals met head to head further forward movement was halted, and animals reciprocally touched each other with their tentacles (Fig. 6B). This interaction was normally very brief, but seemed to be crucial for sperm transfer, which almost always took place only in interactions where a head-to-head position, including tentacle contact, had been attained. The animals then moved slowly towards one another until they began to pass, with the head of each animal moving along the right side of the other (Fig. 6C). This behaviour brings the gonopores into contact.

Immediately prior to penis protrusion the cerata were raised, probably to expose the gonopore and give way for the penis. The huge penes were then simultaneously protruded (Fig. 6D), and each slug deposited a single spermatophore on its partner’s back while withdrawing the penis (Fig. 6E). After the spermatophores were released each slugs extended its penes again, and stroke it over the spermatophore. Shortly after spermatophore exchange the animals separated (Fig. 6F). Most pre-mating interactions did not lead to successful matings, but when a mating occurred spermatophore transfer was virtually always reciprocal and simultaneous. Almost all matings (88%) involved reciprocal exchange of spermatophores. I have never observed any aggressive, or antagonistic behaviours during courtship or mating.

The spermatophore cover hardened shortly after contact with water, and started to dissolve. After spermatophore deposition sperm gathered onto the epidermis and some penetrated into epidermal cells, occasionally causing considerable damage. However, no sperm was observed penetrating the muscle layer beneath the epidermis. Usually the spermatophore was placed with its posterior end close to, or even on, the genital cone. Spermatophores were often partly eaten by the recipient shortly after transfer.
Fig. 6. Behavioural sequence of mating in *A. glauca*. Courtship is initiated by one animal following another (A). The other slug responds by turning around to achieve mutual mouth and tentacle contact (B), and the animals then move slowly forward until the gonopores on each animal’s right side are opposite each other (C). Simultaneous penis eversion (D) and spermatophore transfer follow (E), after which the animals separate (F). Note that both animals are now equipped with spermatophores.

Some time (2-5 h) after sperm transfer a thread of sperm embedded in prostatic fluids emerged from the spermatophore, and started to travel towards the gonopore. Sperm
apparently sought the shortest pathway to the gonopore, which in most cases involved passing between cerata (Fig. 7). Sperm are probably guided to the gonopore chemotactically, and move aided by epidermal ciliation. Depending on the spermatophore’s distance from the genital opening sperm needed up to four hours to complete migration. Sperm uptake may take, in addition, another 2-3 hours. Occasionally slugs interrupted sperm uptake, either by closing the gonopore, or by eating off the approaching sperm string. Sperm viability is probably quite high in *A. glauca*. Sperm taken from an almost totally dissolved, but attached, spermatophore more than 21 hours after mating were still vitally mobile when observed under a light microscope. The prostatic fluids transferred together with the sperm probably provide a protecting and nourishing environment for the spermatozoa.

![Image](image.png)

**Fig. 7.** Sperm migration, video frame. Migrating sperm thread (arrow head) entering gonopore (arrow), about 7 hours after spermatophore transfer.

I found considerable variation in how long spermatophores stayed attached to the receiver after transfer (0-30 h). 29% of the spermatophores fell off immediately, or within four hours after transfer, and were thus probably useless in terms of fertilization. The
remaining spermatophores stayed on for more than five hours. Interestingly, spermatophores transferred in reciprocal matings stayed on for longer than those from unilateral matings.

The place of spermatophore attachment may also vary. Most spermatophores were placed with their posterior ends between cerata rows 3-4 (35%), or rows 4-5 (35%). The remaining sites of placement, where 30% of all spermatophores were placed, ranged between cerata rows 1 and 7. Spermatophores that were placed between rows 3-5 stayed on for longer than those placed elsewhere.

Egg production in *A. glauca* was found to be very high (individuals produced up to 20 clutches per lifetime), and slugs normally produced new clutches every 2-3 days regardless of whether these eggs were fertilized or not. This suggests that slugs were incapable of regulating egg laying, and that eggs, unlike sperm, could be stored over longer periods. Data further indicated that sperm from one mating may not be sufficient to fertilize all eggs.

Not surprisingly I found a highly significant correlation between body size and female fecundity. Size at first reproduction was highly variable, but even very small slugs were able to reproduce, suggesting that the onset of egg production might be influenced mainly be external cues, rather than individual body size.

Overall, *A. glauca*’s mating behaviour contains several intriguing features, which indicate that mate choice takes place. During courtship there are ample opportunities for mate assessment, and following spermatophore deposition the recipient apparently has considerable control over sperm. It therefore seems likely that manipulative behaviours, designed to persuade the partner to keep the spermatophore and use its sperm for fertilization, might have evolved in this species. Sperm penetrating epidermis may be an example of such a mechanism, but it is also possible that the spermatophores themselves function as sexual signals, informing the recipient of the donor’s quality.
Mate choice in *Aeolidiella glauca* (III and IV)

It has been suggested that sexual selection plays an important role also in hermaphrodite mating systems (Michiels 1998). However, sexual conflicts may take unexpected forms, since hermaphrodites are required not only to maximize their male and female success, but they also have to mate with partners and potential rivals at the same time. Many hermaphrodites perform prolonged courtship and complex mating behaviours, which suggests that mate assessment, i.e. pre-copulatory mate choice, may take place.

In addition to pre-copulatory mate choice, post-copulatory mechanisms (e.g. sperm competition) may be a significant selective force in hermaphrodites, particularly as hermaphrodites often occur in high densities, and multiple matings are frequent. With this in mind we might expect different adaptations, aimed specifically at decreasing the impact of sperm competition, to evolve.

In papers III and IV I investigated whether *A. glauca* exhibits pre-copulatory mate choice with respect to body size and presence of spermatophores.

In paper III I performed experiments where slugs in groups of four were allowed to mate freely. Pairs were found to form randomly with respect to body size (Table 1), and size differences within pairs did not differ from those found in natural pairs. In a second experiment I tested whether size is important in competition for partners. In *A. glauca* courting slugs often attract other conspecifics, which may try to break up the original pair. Although partner displacement attempts were frequent, large slugs were not more successful at interfering than smaller slugs. Taken together, these results suggest that size is unimportant with respect to mate choice.

However, pre-copulatory mate choice may still take place. I performed an experiment where mate choice was restricted and found that mating latency increased, whereas the proportion of reciprocal matings decreased. Further, I found that the presence of a spermatophore reduced a slug’s ability to interrupt matings. These results suggest that mate choice based on traits other than size, e.g. presence of spermatophore, might take place.
Table 1. Pair formation in groups consisting of four slugs. Pairs of different size composition were formed in frequencies expected under random mating ($\chi^2 = 4.4$, $p = 0.2$).

<table>
<thead>
<tr>
<th>Pair information</th>
<th>Expected matings</th>
<th>Observed matings (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S - M</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>S - L</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>M – M</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>M – L</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

In paper IV the effect of spermatophore presence on mate choice in *A. glauca* was further investigated. Mate choice trials involving three slugs were performed, where I found that slugs carrying spermatophores were avoided as partners, and thus less likely to participate in matings than other slugs (Fig. 8).

![Fig. 8](image)

**Fig. 8.** Frequency of different matings in mate choice trials. More matings occurred between animals without spermatophores (hatched bar), than there were matings involving spermatophore-carrying slugs (grey bar) (Binomial, $p = 0.05$).
Interestingly, this mate choice behaviour was not influenced by current mating status. Isolated animals, that presumably had been able to build up sperm reserves, also refrained from mating with conspecifics already carrying spermatophores (Fig. 9).

**Fig. 9.** Frequency of matings in mate choice trials with isolated animals. More matings took place between animals without spermatophores (hatched bar), than there were matings involving spermatophore carriers (grey bar) (Binomial, p = 0.012).

The most obvious explanation for *A. glauca*’s intriguing spermatophore avoidance behaviour is that it reduces the risk of sperm competition. Another interpretation is that by avoiding partners with spermatophores, slugs decrease the risk of getting a partner depleted in autosperm. However, as almost all animals had sufficient sperm in store for spermatophore production, I find this explanation unlikely. Thus, this study provides a unique example of hermaphrodite mate choice behaviour, which may have evolved as a response to potential sperm competition.
Sex allocation in *Aeolidiella glauca*

According to the prediction derived from sex allocation models (Charnov 1996; Greeff & Michiels 1999b), an increased mating rate should result in an increased allocation to the male function, whereas the opposite is true for the female function. In paper V I experimentally tested whether this prediction holds true for *A. glauca*. I divided slugs in three groups with differing mate encounter rates (high, medium and low), and then compared egg and spermatophore production between these treatments.

As expected, slugs most frequently introduced to potential partners mated more often and laid significantly fewer eggs compared to slugs with fewer mating opportunities (Fig. 10).

![Fig. 10](image) **Fig. 10.** Bars illustrate the mean (± s.e) egg production in the different treatments. Slugs that were introduced to partners most frequently (high) produced fewer eggs than others. Significant differences in post-hoc tests are illustrated with bars, ** p < 0.01 (MANCOVA: F_{2,63} = 3.84; p < 0.05).**

Slugs with more mating opportunities also produced proportionately more spermatophores than those presented with medium and low numbers of partners, as a direct consequence of participating in more matings (Fig. 11). This experiment showed that *A. glauca* is clearly able to adapt to different mating opportunities by altering its relative
reproductive output. This is in concordance with earlier studies of sex allocation in simultaneous hermaphrodites (Raimondi & Martin 1991; Trouvé et al. 1999), but contrasts to a study of the landsnail *Arianta arbustorum* (Locher & Baur 2000), where overall reproductive allocation remained highly female biased despite differing mating frequencies.

![Figure 11](image-url)

**Fig. 11.** Bars illustrate the mean (± s.e) sperm production in the different treatments. Slugs introduced to partners most frequently (high) had higher sperm production than others. Significant differences in post-hoc tests are illustrated with bars, *** p < 0.001 (MANCOVA: F₂,₆₈ = 8.29; p < 0.001).

Previous work on hermaphrodite sex allocation have primarily focused on lifetime allocation to male and female function depending on the size of mating groups (Raimondi & Martin 1991; Trouvé et al. 1999), or the degree of outcrossing (Wedekind et al. 1998; Weinzierl et al. 1998; Schärer & Wedekind 2000). In contrast this study provides a unique example of a simultaneous hermaphrodite that can adapt quickly to current mating conditions by regulating the reproductive output via its male and female function. Like many other nudibranch species *A. glauca* probably experiences fluctuating population densities, which means that short term flexibility could be of great value.

**GENERAL CONCLUSIONS**
This thesis has investigated the mating system and reproductive biology of the simultaneously hermaphroditic nudibranch *Aeolidiella glauca*. I have shown that both the genital system (I) and mating behaviour (II) differs from that previously described for this species. I found that *A. glauca* exhibits a unique mating behaviour, with external and reciprocal transfer of spermatophores. This behaviour contains several intriguing features, suggestive of post-copulatory mate choice based on spermatophore and/or sperm quality.

The finding that *A. glauca*’s external spermatophores may play an important role in mate choice is supported by results from papers III and IV. Here I found that slugs already carrying spermatophores were less likely to succeed when competing for mates (III), and that they were often avoided as partners (IV). The most parsimonious explanation for this unique mate choice behaviour is that it reduces the risk of sperm competition: spermatophores provide visual evidence that a slug has recently mated and received sperm.

In addition to active mate choice hermaphrodites may increase their reproductive success by differential allocation to the male and female functions. In paper V it was shown that *A. glauca* flexibly allocated resources to the male or female function in response to differing mating opportunities. To my knowledge, this it the first example of a hermaphrodite that can adapt to short term fluctuations in partner availability by regulating its reproductive output in an opportunistic way.

To date, there are few studies of simultaneous hermaphrodites in the context of sexual selection. *Aeolidiella glauca* may, with its external sperm transfer, prove to be a study organism particularly well suited for studies within this field. Moreover, the external spermatophores are also useful in sex allocation studies, as they provide a direct measure of current reproductive output.
TACKNOWLEDGEMENTS

Writing this thesis has been much like participating in a theatre play. Many different characters are involved, and they’re all needed to perform their unique and important roles. The leading actors in this particular play are of course my little naked friends, to whom I am heavily indebted. They have kindly co-operated (?) despite my efforts to broadcast some of their most intimate secrets. However, I have this nagging suspicion that I’ve only scratched a little bit on the surface yet, and that the real show goes on without me…

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I’m getting into a very sentimental mood now, and it’s high time to draw the curtains.
This play may be over, but the show must go on and I hope to see you all in Gothenburg soon!

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Nakensnäckan *Aeolidiella glauca* - en hermafrodit med många hemligheter.


Inom biologin tänker vi inte så mycket på hermafroditernas gudomliga ursprung, utan definierar istället hermafroditer som organismer med både hanliga och honliga könsorgan. De flesta växter är hermafroditer med både pistill och ståndare i samma blomma. Bland djur förekommer hermafroditer i alla större djurgrupper utom fåglar, reptiler och däggdjur. Särskilt vanligt är det hos blötdjur där omkring 40% av alla släkten innehåller hermafroditiska arter.


Simultana hermafroditer fungerar istället som hane och hona på samma gång. En del simultana hermafroditer kan befrukta sig själva, men de flesta behöver en partner att para sig med.

Trots att det är relativt vanligt med simultan hermafroditism så har det bedrivits mycket lite forskning kring detta reproduktionssätt, och många grundläggande frågor återstår att besvara. Kunskapen om enskilda arters reproduktion är också bristfällig, och de redogörelser som gjorts är ofta ganska ofullständiga. Det blev jag snart varse då jag påbörjade mina studier av *A. glauca*. Den enda tidigare beskrivning som gjorts av arten visade sig innehålla en mängd felaktigheter, både vad gäller anatomi och beteende, och jag blev således tvungen att börja ändå från början.

I kapitel I beskrivs hur könsorganen hos *A. glauca* är uppbyggda, och i kapitel II ges en utförlig beskrivning av snäckans parningsbeteende. Det visade sig att *A. glauca* har ett ganska komplicerat inre, och att parningsbeteendet innehåller flera unika drag. Till exempel parar sig inte snäckorna i vanlig bemärkelse, utan spermierna överförs istället på utsidan av kroppen, i ett slags spermiepaket som “klistras” fast på mottagarens rygg. Några spermier tränger därefter ner i huden på mottagaren, och når i vissa fall djupt ner i de underliggande muskellagren. De når dock aldrig fram till äggen, så dessa spermier deltar således inte i befruktningen. Merparten av spermierna “läcker” dock ut ur spermiepaketet, som successivt luckrats upp, och påbörjar sin mödosamma vandring längs mottagarens hud. Efter åtskilliga
timmar när spermierna sitt mål, könsöppningen, där de kryper in. Det är alltså först nu som spermierna befinner sig inne i de reproduktiva organen hos mottagaren, och kan befrukta dess ägg. Men vägen dit är ingalunda smärtfri. Spermier mottagaren kan nämligen ta till en mängd olika knep för att förhindra att spermierna når fram till äggen. Till exempel kan den helt sonika stänga sin könsöppning, eller till och med äta upp de annalkande spermierna!


I de flesta parningar (88%) överför båda djuren samtidigt ett spermiepaket, men när möjligheten till partnerval försvann så minskade andelen sådana parningar betydligt. Det visade sig också att spermiepaketet hade en avskräckande verkan; de djur som redan hade parat sig och fortfarande bar på ett spermiepaket var inte längre attraktiva för andra. Detta medförde att de hade svårare att konkurrera om partners, och ofta ratades till förmån för andra snäckor. Det finns flera tänkbara skäl till varför snäckor med spermiepaket valdes bort, men den troligaste är kanske att sådana snäckor har färre obefruktade ägg kvar. En snäcka som bär på ett spermiepaket har bevisligen parat sig nyligen, och kan således redan ha befruktat alla sina ägg. Genom att undvika en sådan snäcka och istället satsa på någon som inte parat sig ökar en parningssugen snäcka sina möjligheter att få många avkommor.

Det som gör hermafroditer lite knepigare än andra djur är just det att de kan vara både hane och hona på samma gång. Det betyder dock inte att de alltid är det, eller att de är båda delarna hela tiden. Jag ville veta om *A. glauca* kan växla mellan sin hanliga och honliga funktion, och gjorde därför ett experiment (kapitel V) där några snäckor fick möjlighet att para sig ofta, medan andra istället fick få parningsmöjligheter. Jag jämförde sedan produktionen av ägg och spermiepaket mellan grupperna för att se om de som fick para sig ofta lade mer energi på att tillverka spermier än de som fick färre parningsmöjligheter. Det visade sig att snäckor som parat sig oftare tillverkat fler spermiepaket, medan de som parat sig mer sällan istället tillverkat fler ägg. Det verkar således som om *A. glauca* kan vara ganska flexibel ifråga om vilken könlig funktion den antar. Då det finns gott om tänkbara partners, och goda möjligheter att reproduera sig som hane satsar snäckorna mer resurser på detta, men om möjligheterna till parning minskar så skjutsas resurserna istället över till den honliga funktionen. Hos många nakensnäckor är det vanligt med snabba och stora förändringar av antalet individer i en population, och förmågan att snabbt kunna anpassa sig till förändrade förhållanden är säkerligen mycket värdefull. Det skulle till och med kunna vara så att hermafroditer är hermafroditer just för att de lever i snabbt förändringliga miljöer där det är särskilt viktigt att vara flexibel.
I den här avhandlingen har jag belyst några aspekter av reproduktionen hos en simultan hermafrodit. Jag är dock övertygad om att “min” art, liksom många andra, fortfarande ruvar på många hemligheter, och jag hoppas därför att många i framtiden ska få upp ögonen för hermafroditer och deras fascinerande, till stor del ännu outforskade liv.