

Thesis for the degree of Doctor of Philosophy

MATE CHOICE AND ITS EVOLUTIONARY
CONSEQUENCES IN INTERTIDAL SNAILS
(*LITTORINA* SPP.)

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*Till
Thomas och Sebastian
som alltid står vid min
sida och utan vilkas
stöd jag inte
står*

ABSTRACT

The ability to recognise potential mates and choose the best possible partner for reproduction is of fundamental importance for most animal species. This thesis presents studies of mate choice in marine snails of the genus *Littorina*, where male precopulatory mate choice seems to be more predominant than female precopulatory choice. Male mate choice starts already when the male choose (or not) to follow another individual's mucus trail, which is deposited during locomotion. Trail-following is a central part of this thesis and gastropod trail-following in general has been thoroughly reviewed in one of the chapters. Both trail-following and copulations have been studied in several experiments to depict male mate choice and the consequences of mate choice on reproductive barriers between ecotypes (of *L. fabalis*) and sister-species (*L. fabalis* and *L. obtusata*).

Mate choice does not always stop with a completed copulation. In species where females mate with multiple males there is a chance for cryptic mechanisms to act after copulation through cryptic female mate choice and/or sperm competition. In one of the chapters paternity distribution among offspring of females of *L. saxatilis* was studied, using genetic tools, and laboratory-raised families with known parents. Paternity was not randomly distributed among the fathers contributing to offspring production, indicating postcopulatory sexual selection either by cryptic female choice and/or sperm competition.

Convenience polyandry has been suggested as an explanation for the apparent lack of female precopulatory mate choice and the extreme promiscuity in *L. saxatilis*. In this species we found that females try to reduce the number of costly matings by removing cues from their mucus trails, to avoid advertising their sex as females of closely related species do. Thus males are forced to search blindly for mates following male- and female mucus trails indiscriminately, consequently reducing male-female encounters and costs of superfluous matings for the female.

This thesis contributes to new insights on mate choice and its effects on reproductive barriers in *Littorina*, and adds to our understanding of the evolution of new species.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Förmågan att känna igen potentiella partners och välja den bästa partnern att reproducera sig med är av fundamental vikt för de flesta djur. Den här avhandlingen presenterar studier av partnerval hos strandsnäckor (*Littorina* spp.), där hanligt val är mer framträdande än honligt val. Hanarna gör sitt första val redan när de väljer att följa eller inte följa en annan individs slemspår. Slemspår produceras av alla snäckor när de rör sig och utgör en förutsättning för snäckors förflyttning. Slemspårföljning är en central del av den här avhandlingen och i ett av kapitlen ges en översikt över slemspårföljningens betydelse hos snäckor mer generellt. Både slemspårföljning och parningar har studerats i flera experiment där fokus har varit på hanligt val och dess konsekvenser för parningsbarriärer mellan bestånd av en art (ekotyper) och arter.

Partnerval är inte alltid så enkelt som att se vem som parar sig med vem, hos honor som parar sig med många hanar finns möjlighet för partnerval även efter parningen; antingen genom honlig påverkan på befruktningprocessen eller genom att spermier från olika hanar konkurrerar om befruktningen. I ett av avhandlingens kapitel studeras hur faderskap fördelas mellan olika tänkbara hanar hos snäckfamiljer uppfödda på labb. Vi fann att faderskapsfördelningen inte var slumpmässig bland de hanar som bidrog till kullarna, vilket tyder på att det förekommer ett urval efter parning antingen genom honlig påverkan och/eller genom spermiekonkurrens.

Avsaknaden av ett synligt honligt val hos strandsnäckor tros bero på att kostnaden för att neka hanarna parning är högre än kostnaden för honan att acceptera en parning, så honorna väljer att acceptera alla hanar snarare än att försöka mota bort oönskade hanar. Detta innebär att strandsnäckor parar sig ofta och har många fäder till varje kull ungar. Hos den mest promiskuösa arten (*L. saxatilis*) fann vi att honorna försöker att minska antalet parningar på ett oväntat sätt. Medan andra snäckarters honor märker sina slemspår, för att locka hanar till sig, gör *L. saxatilis* honorna inte detta. Deras spår blir då identiska med hanarnas egna spår och detta förvirrar hanarna, som måste följa både honor och hanars spår för att hitta en partner. Därigenom minskar antalet parningar eftersom hanarna är upptagna med att följa varandras spår hälften av gångerna.

Den här avhandlingen bidrar till nya kunskaper om partnerval hos strandsnäckor och hur detta påverkar parningsbarriärer mellan populationer. Den bidrar därmed till att öka vår förståelse om hur evolutionen bildar nya arter.

LIST OF PAPERS

This thesis is a summary of the following papers, which will be referred to in the text by their Roman numbers:

Paper I: Terence P. T. Ng, Sara H. Saltin, Mark S. Davies, Kerstin Johannesson, Richard Stafford, Gray A. Williams (2013). Snails and their trails: the multiple functions of trail-following in gastropods, *Biological Reviews*, doi: 10.1111/brv.12023.

Paper II: Sara H. Saltin, Hanna Schade, Kerstin Johannesson (2013). Males' preference for large females cause a partial mating barrier between a large and a small ecotype of the marine snail *Littorina fabalis* (W. Turton, 1825), *Journal of Molluscan Studies*, **78**: doi:10.1093/mollus/eyt003.

Paper III: Sara H. Saltin, Eva-Lotta Blom and Kerstin Johannesson (Manuscript). Male mate preference in conflict with species recognition in a trail-tracking gastropod.

Paper IV: Sara H. Saltin, Gregory Charrier, Marina Panova, Anna-Karin Ring, Carl André, Kerstin Johannesson (Manuscript). Postcopulatory sexual selection in a highly promiscuous snail (*Littorina saxatilis*).

Paper V: Kerstin Johannesson, Sara H. Saltin, Iris Duranovic, Jon N. Havenhand, Per R. Jonsson (2010). Indiscriminate males: mating behaviour of a marine snail compromised by a sexual conflict? *Plos One*, **5**: e12005.

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INTRODUCTION

Mate choice

From an evolutionary perspective, mate choice can be the most important decision in an individual's life, at least for sexually reproducing animals. Some animals spend their whole life with one partner or share the responsibility of raising the offspring, while most animals (not least invertebrates) meet their partner only briefly during mating. Either way, choice of mate is fundamental since the quality of the mate is likely to affect the fitness of the choosing individual through direct or indirect benefits. Direct benefits are, for example, nuptial gifts that is transferred during the copulation (Wedell and Ritchie 2004), and other benefits such as parental care (Clutton-Brock 1991) or protection/acceptance within a territory (Koenig and Dickinson 2004). Indirect benefits of mate choice are benefits that have fitness effects for the choosing individual by influencing offspring's survival or attractiveness (Møller and Alatalo 1999). Indirect benefits should be much more wide spread since all sexually reproducing animals should choose the mate that ensures the best fitness of the offspring, as long as the costs of the choice do not exceed the benefits of the choice (Jennions and Petrie 1997).

Although recent studies have pointed out the importance of male mate choice (Vincent, Ahnesjö et al. 1992; Bonduriansky 2001; Edward and Chapman 2011), or mutual mate choice (Myhre, de Jong et al. 2012; Kvarnemo and Simmons 2013), studies of mate choice have traditional focused on female choice. The reason for this bias is the preconception that a female's reproductive capacity is limited by the number of eggs and that females therefore are choosy, while males mate at every possible occasion since their reproductive capacity only are limited by the number of available females (Darwin 1871; Bateman 1948; Williams 1975; Dawkins 1976). However, numerous studies have shown that males as well as females often have costs of mating and that their reproductive capacity is restricted by other factors than the availability of females (Daly 1978; Dewsbury 1982; Michener and Locklear 1990; Wedell, Gage et al. 2002; Jordan and Brooks 2010). Indeed, indiscriminate mating should occur only if the benefits outweigh the costs of mating, and consequently both

males and females tend to be choosier about their mates in proportion to how much time and energy they invest in mating (Ridley 1983).

Male mate choice is often obvious from behavioural observations of courting and mating. However, female mate choice is not always easily unveiled since females that mate more than one male have a potential for mate choice after copulation, through cryptic female choice (Eberhard 1996).

Mate choice and mate competition through sexual selection (Andersson 1994) have given rise to many of the most remarkable phenotypic characters we can find in nature, for example, the tails of the peacocks (Darwin 1871) and the widowbirds (Andersson 1982), and extensive colouration and body-shape in families of teleost fishes (Kodric-Brown 1985; Quinn and Foote 1994). Less obvious, but nonetheless important is the adaptations to postcopulatory sexual selection, as a way for males to increase fertilization success during sperm competition or cryptic female choice (Kvarnemo and Simmons 2013). During the evolution of species, sexual selection has been a major mechanism influencing morphology, physiology and behaviour in many species (Andersson 1994). Moreover, divergent sexual selection within a population can lead to reproductive isolation and speciation (Seehausen, Terai et al. 2008). Mate choice and reproductive barriers are intimately connected given that the fundamental and obvious strategy in mate choice must be to mate with individuals of the opposite sex and of the same species. To achieve this, mate recognition can sometimes be a complex process involving several cues and stimuli (Hankison and Morris 2003). This complexity serves to prevent interspecific matings with the risk of producing inferior hybrids (Coyne and Orr 2004). However, as will be presented in this thesis, mate choice can sometimes be further complicated by conflicting interests of preferable traits and species recognition (Pfenning 1998) or by sexual conflicts (Arnqvist and Rove 2005).

The study system of Littorina

Gastropods are one of the most diverse groups of animals and it can be divided into three subclasses: the prosobranchs, opisthobranchs and pulmonates (Haszprunar 1988). Most opisthobranchs and all pulmonates are hermaphrodites, while 97% of the prosobranchs are gonochorists and thus have separate sexes (Baur 1998). As most other prosobranchs, all 19 species of the periwinkles, genus *Littorina*, have separate

sexes and they practice internal fertilisation (Reid 1996). *Littorina* snails are widely spread in the subarctic and temperate regions of the Northern Hemisphere (Reid 1996). They all inhabit the littoral-zone, but some species live mostly submerged on seaweeds or rocks, while other species are confined to rocks above mean-water and up into the splash-zone (Reid 1996). Snails in the upper littoral zone can have long intervals of inactivity, sometimes being dried up for weeks during summer (Stafford & Davies 2004). When wetted they move by muscular movements in the foot, gliding on secreted mucus that is deposited as a trail. This way of movement enables them to adhesive to the substratum and perform food- and mate search even during heavy wave action and on vertical or overhang surfaces.

In Sweden there are four species with different life history traits: *Littorina littorea*, *L. saxatilis*, *L. fabalis* and *L. obtusata* (Fig. 1; Table 1). The most distantly related species of the four is *L. littorea* (Reid, Dyal et al. 2012), which have a different mode of reproduction as it has a planktotrophic development and spawn during winter and spring (Reid 1996). *Littorina saxatilis* is ovoviviparous and is reproductively active year-round, while *L. fabalis* and *L. obtusata* have a somewhat more restricted mating season with egg-masses being mostly observed during late spring and summer (pers. obs.). Densities of Swedish populations of the four species vary a lot (Table 1) providing different potentials for mate interactions in different species.

Littorina fabalis and *L. obtusata* are sister-species that separated about 1.3 Mya (Tatarenkov 1995; Reid, Rumbak et al. 1996; Reid, Dyal et al. 2012). These species are similar in shape, but adult *L. obtusata* are larger than *L. fabalis* and the former also lives several years while the latter lives only one to two years (Williams 1990). The only diagnostic differences in morphology is found in the genitals (Reid 1996) and in addition there are several diagnostic allozyme loci (Zaslavskaya, Sergievsky et al. 1992; Rolán-Alvarez, Zapata et al. 1995; Tatarenkov 1995). The two species have a partially sympatric distribution on wave-protected shores in the NW Atlantic as well as overlapping reproductive seasons (Reid 1996) and, in Sweden, the two species are occasionally observed to engage in interspecific copulation (Saltin pers. obs.). Nevertheless, according to genetic data the species do not currently hybridise, though they share most of the common mitochondrial haplotypes, suggesting either that hybridization has taken place after their first separation, or that the two species still share ancestral mtDNA haplotypes owing to incomplete lineage sorting (Kemppainen, Panova et al. 2009).

Both *L. saxatilis* and *L. fabalis* are polymorphic species with ecotypes, adapted to different environments, and forming often narrow hybrid zones in between. The ecotypes of *L. saxatilis* in Sweden comprise the smaller and more fragile exposed ecotype (E) living on wave-exposed cliffs and the larger and more robust sheltered ecotype (S) living in micro-sheltered environments behind and beneath boulders (Janson 1982). *Littorina fabalis* lives on furoid and other macroalgae. In Sweden, the “small-sheltered” ecotype (SS) inhabits sheltered parts of shores, while the “large-moderate” ecotype (LM) is confined to moderately exposed parts of shores (Kempainen, van Nes et al. 2005). In both species, ecotypes differs in size, while in *L. saxatilis* there are also differences in morphology and shell thickness, which are largely inherited (*L. saxatilis*: Janson 1982; Janson and Ward 1984; Reid 1996; Johannesson and Tatarenkov 1997, *L. fabalis*: Reimchen 1982; Reid 1996; Tatarenkov and Johannesson 1998; Kempainen, van Nes et al. 2005). While there are extensive reproductive barriers due to size assortative mating between the ecotypes of *L. saxatilis*, which reduce the gene-flow to 10-30% in the hybrid zone (Johannesson, Rolán-Alvarez et al. 1995; Rolan-Alvarez, Erlandsson et al. 1999; Grahame, Wilding et al. 2006; Panova, Hollander et al. 2006), the gene-flow between the ecotypes of *L. fabalis* is only slightly reduced (Tatarenkov and Johannesson 1998). Also *L. obtusata* can be polymorphic in parts of its range (Reid 1996), but the populations in Sweden do not unveil distinct ecotypes.



Figure 1. The four species of *Littorina* that occur in Sweden, (A) *L. littorea*, (B) *L. saxatilis*, (C) *L. fabalis* and (D) *L. obtusata*. Photo: Patrik Larsson (A-C) and Sara Hintz Saltin (D).

Species	Reproductive season	Number of eggs per year	Snail density ¹
<i>L. saxatilis</i> ²	Year round ³	200 ⁴	280
<i>L. littorea</i>	February-June ³	110.000 ⁵	2.3
<i>L. fabalis</i>	March-September ³	>600 ⁴	1.4
<i>L. obtusata</i>	March-September ³	>500 ⁴	1.1

Table 1. ¹Snail density estimates are from typical habitats of each species taken into account the surface area of the substratum, see Paper II for details. ²Densities for *L. saxatilis* are indicated for the Swedish exposed ecotype (E). ³Data on reproductive seasons are according to Reid 1996 and personal observations of the local conditions in Sweden and ⁴number of eggs per year are from unpublished studies by Johannesson K except for ⁵*L. littorea* where data are from (Buschbaum and Reise 1999).

Reproductive behaviour in snails

There is strong evidence for high levels of promiscuity in several species of gastropods (Baur 1998), and species of the genus *Littorina* are no exception. Indeed, there are high- to extremely high levels of multiple paternity in two species, with five sires per brood in *L. obtusata* and as many as 10-20 sires per brood in *L. saxatilis* (Paterson, Partridge et al. 2001; Mäkinen, Panova et al. 2007; Panova, Boström et al. 2010).

In *Littorina* it is the males that are active in finding a mate and in initiating copulation, while females are seemingly passive. Indeed, females that attempt to actively reject males have only been observed on a few occasions in *L. littorea* (Saur 1990; Erlandsson and Johannesson 1994). That females would completely lack the ability to choose a male to mate with is quite remarkable given the extreme promiscuity in *L. saxatilis* (Panova, Boström et al. 2010). Possibly females have limited means to reject an approaching male since fleeing or fighting off males seem unlikely or too costly. To escape by withdrawing into the shell would be risky since this would cause dislodgement from the substratum with a high risk of being swept away by waves. Hence, due to these high costs of resisting males, it has been proposed that females accept more or less all mating attempts since the costs of superfluous mating is likely to be lower than the costs of fighting off males (convenience polyandry) (Thornhill and Alcock 1983; Panova, Boström et al. 2010).

For males, the first step in mate choice is taken already when the male choose (or not) to follow a mucus trail (Fig. 2.1) (**Paper I**) of a female. Notably, males of some species (*L. littorea*, *L. fabalis* and *L. obtusata*) are able to detect gender identity

already in the mucus trail (**Paper V**). Hence, males are able to selectively track females in search of a mate. Moreover, males can also make female-quality assessments from the mucus trails and they mostly prefer trails from large and more fecund females (**Paper II and III**). When encountering a female, males may choose to mount the females' shell (Fig. 2.2) and start to explore it during circular counter-clockwise movements (Saur 1990). The mounting phase may lead to copulation (Fig. 2.3), or is terminated by the male leaving the female. In *Littorina*, mating time is usually 10-60 min, while shorter "copulations" are observed when males initiate mating with improper mates (such as another male or a juvenile) (Saur 1990; Erlandsson 1998).

In species that mate repeatedly within the same mating season, sperm from several males is present in the female during fertilization. Hence there is a potential for postcopulatory sexual selection. Patterns of non-random paternity distribution in broods of *L. saxatilis* indicate that there are postcopulatory selection processes influencing paternity in this species (**Paper IV**). This could be male driven by sperm competition (Birkhead and Moller 1998) and/or female driven by cryptic female choice (Eberhard 1996) (Fig. 2.4).

Four stages of mate choice:				
Choices:	Follow trail or not? If yes! ↓ How long (distance)?	Mount or not? If yes! ↓ How long (time spent)?	Copulate or not? If yes! ↓ How long (time spent)?	Cryptic female choice? ?? Sperm competition?
Studied in:	Papers I, II, III and V	Paper III	Papers II and III	Paper IV

Figure 2. Four possible stages of mate choice in *Littorina*: (1) trail-following, (2) mounting, (3) copulation and (4) postcopulatory sexual selection.

TOPICS OF THE INCLUDED PAPERS

Trail-following

As gastropods move by muscular movements in the foot and glide on mucus, they leave a trail of mucus behind. It has been known for decades that gastropods often choose to follow their own or other individuals' trails (Paine 1963; Cook, Bamford et al. 1969; Hall 1972). In **Paper I**, we reviewed over 40 years of research and found that trail-following can have multiple functions across and within genera: (1) homing, (2) nutrition and energy conservation, (3) self-organisation and aggregation, and (4) mate location and communication.

1. **Homing:** In gastropods the behaviour of “homing” is defined as returning to a resting position after an active period, typically after a feeding excursion, which sometimes (e.g. in some limpets) is an individually marked position on the rock known as a “home scar” (Cook 1969; Branch 1975; Chelazzi, Focardi et al. 1989). Finding the way home can be mediated by trail-following and some homing species can even recognise their own trail and follow it back after foraging (Cook 1992). However, homing species does not always rely on mucus trails to find their way back, since there are other mechanisms for navigation (e.g. air-born chemical cues) that can be used instead of or in addition to trail-following (Cook 1980).
2. **Nutrition and energy conservation:** Mucus trails can be used for feeding, either by trail-following to ingest trapped or growing organic material in the trails (Peduzzi and Herndl 1991; Davies and Beckwith 1999; Coffin, Barbeau et al. 2012), or as a way for predators to locate gastropod prey (Pearce and Gaertner 1996; Clifford, Gross et al. 2003; Iwai, Sugiura et al. 2010; Lai, Chen et al. 2011). It has also been shown that snails that move in a previously laid trail can use less force during locomotion and need to deposit less amounts of mucus, making trail-following less energetically costly than normal locomotion (Tankersley 1989; Davies and Blackwell 2007).
3. **Self-organisation and aggregation:** Following mucus trails allows self-organisation in several groups of gastropods, since this facilitates aggregation. This is especially common in intertidal littorinids that follow the tidal cycle to

forage in moist conditions and find shelter by aggregating in dry conditions (Garrity 1984; Chapman and Underwood 1996).

4. Mate location and communication: Following the trails of conspecifics is common in opisthobranchs and pulmonates and it has been linked to mate-search since trail-following often results in courtship (Cook 1977; Leonard and Lukowiak 1985; Wareing 1986). Moreover, it has been demonstrated that the freshwater pulmonate, *Biomphalaria glabrata*, track conspecifics to a higher degree when sexually aroused (Townsend 1974). Species-specific trail-following and preference to track females trail (rather than males trails) has also been reported in gonochoric prosobranchs (Trott and Dimock 1978; Erlandsson and Kostylev 1995; Ng, Davies et al. 2011; **Paper V**), which suggests that mate search by trail-following is likely to be common in gastropods. To advertise the sex in the mucus trail is probably optimal for females of most species, to attract males. However, as we show in **Paper V**, females of a high-density species disguise their gender identity in the mucus trails to reduce mating intensity. Although possible in theory, very few studies have examined whether or not males can make an assessment of female quality from mucus trails. However, males of *L. littorea* detect and avoid trails of females that have low fecundity because they are parasitized or starved (Erlandsson and Kostylev 1995; Edwards and Davies 2002). In gastropods, quality and female size are linked since large females generally are more fecund (Hughes and Answer 1982; Janson 1985; Erlandsson and Johannesson 1994; Dewitt 1996; Yusa 1996; Angeloni 2003). In **Paper II** and **Paper III** we show that there is a preference for large trails, at least when snail size differences are large enough to be detected from the mucus trails.

In **Paper I** we also reviewed the little that is known about the mechanisms of trail-following: how snails detect mucus trails and the cues in them (such as polarity, species cues, gender cues and individual specific cues) and what these cues consist of. Most likely snails often detect mucus cues by their tentacles, which is supported by observations that snails frequently touch the mucus trail with their tentacles while performing trail-following (Erlandsson and Kostylev 1995; Ng, Davies et al. 2011). Experimental work also shows that the tentacles are important during trail-following since snails with amputated tentacles are hindered (Chase and Croll 1981). However,

one study shows that the buccal lip extension in predatory terrestrial snail *Euglandina rosea* is more important than the tentacles during trail-following (Cook 1985), while in other species trail detection via the foot have been proposed (Paine 1963).

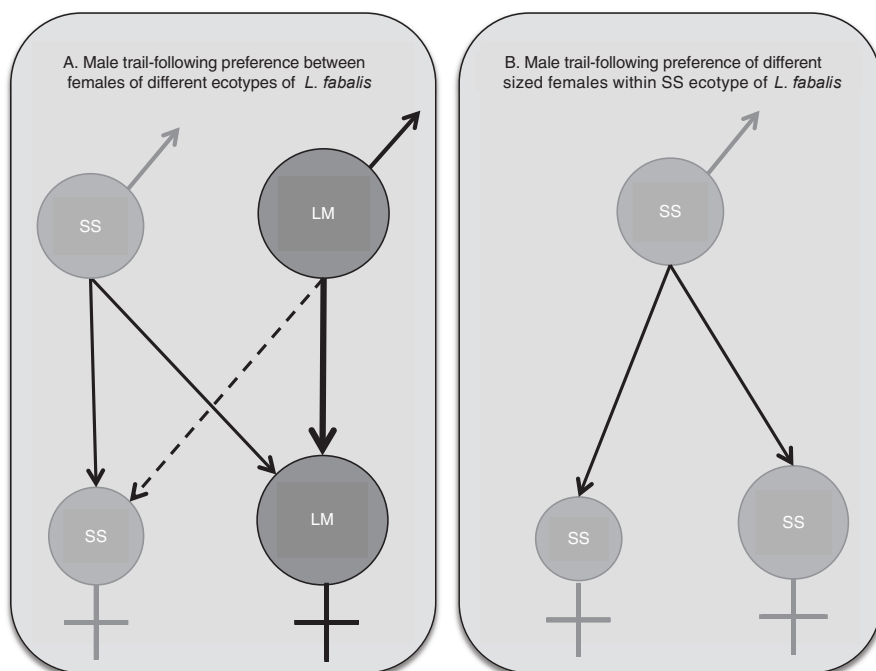
The nature of cues that is imbedded in the mucus trails have often been proposed to be chemical as intraspecific communication via pheromones seem to be most likely (Ng, Davies et al. 2011; **Paper V**). Moreover, species specific chemical composition of trail mucus has been found, although the connection to trail-following behaviour have not been investigated (Smith 2002; Li and Graham 2007). A few studies have shown that substances from mucus trails can trigger trail-following, which in one case was found to be a volatile low molecular weight substance (Ushadevi and Krishnamoorthy 1980) and in another case small and highly water soluble molecules (Clifford, Gross et al. 2003). Another possibility is that there are physical or structural elements in the mucus trails that can be detected and function as information cues (Bretz and Dimock 1983).

Size matters and ecotypes

Body size can play an important role in mate recognition and many animals mate assortatively with respect to size (Ratcliffe and Grant 1983; Richmond and Reeder 2002; Jones, Moore et al. 2003). For example, sympatric species pairs of threespine stickleback (*Gasterosteus* spp.) living in the same lake but adapted to different microhabitats, mate assortatively and only interbreed when sizes overlap (Nagel and Schluter 1998; Van Dongen, Matthysen et al. 1998). A similar situation has been reported among ecotypes of *L. saxatilis*, where snails mate assortatively with respect to size and thereby maintain a partial reproductive barrier between ecotypes that differ in size ranges (Saur 1990; Erlandsson and Rolán-Alvarez 1998; Pickles and Grahame 1999; Cruz, Carballo et al. 2004; Hollander, Lindegarth et al. 2005; Johannesson, Havenhand et al. 2008). In contrast, in other species of *Littorina* sexual selection favouring large females have been found, which likely is explained by the positive correlation between size and fecundity (Hughes and Answer 1982; Janson 1985; Saur 1990; Erlandsson and Johannesson 1994; Rolán-Alvarez, Zapata et al. 1995; Zahradnik, Lemay et al. 2008).

In **Paper II** we studied size and ecotype preference during trail-following and copulation in *L. fabalis*. We found that the large ecotype (LM) showed a preference

for females of their own ecotype both during trail-following and copulation (Fig. 3A and C). Males of the smaller ecotype (SS) showed no clear preference between females of different ecotypes during trail-following (Fig. 3A), although they tended to copulate more with LM females (Fig. 3C and Fig. 3 in **Paper II**). The reason for this skewed mate preference between the ecotypes could be due to a size preference. If males of both ecotypes prefer females that are the same size or larger than themselves, this will result in ecotype assortative mating for LM males while SS males would mate with females of both ecotypes. Indeed, we also found a clear size preference within ecotype, as SS males copulated more with large SS females when choosing between large and small SS females (Fig. 3D). They showed however no preference for different sized mucus trails when choosing between trails from large and small SS females (Fig. 3B), possibly due to difficulties of making an accurate size assessment from trails. In summary, there is an overall preference for copulating with large females in *L. fabalis*, while size preference is weaker at the stage of trail-following.



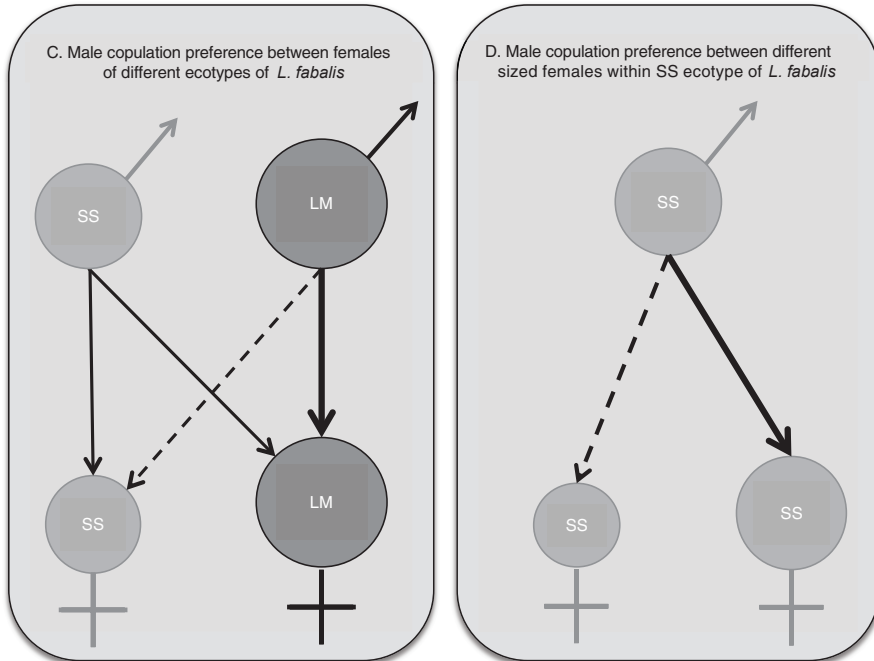


Figure 3. Mate preference in SS and LM ecotypes of *L. fabalis* during trail-following (A and B) and copulation (C and D). Preference is indicated by the thickness of arrows: equally thick arrows show that there is no clear preference for either female while thick arrow indicate significant preference for one group of females.

In hybrid zones in nature, this skewed mating preference likely results in a weak mating barrier between the ecotypes of *L. fabalis* that could account for a previously found slight decrease in gene flow between the two ecotypes (Tatarenkov and Johannesson 1998). Notably, the weak mating barrier between the two ecotypes of *L. fabalis* contrasts with observations made in the closely related species *L. saxatilis*. In *L. saxatilis* mating is strongly assortative with respect to ecotype and males of both the small and the large ecotypes prefer females of their own ecotype over females of the other ecotype (Johannesson, Rolán-Alvarez et al. 1995; Pickles and Grahame 1999; Rolán-Alvarez, Carballo et al. 2004; Hollander, Lindgarth et al. 2005; Grahame, Wilding et al. 2006; Panova, Hollander et al. 2006; Johannesson, Havenhand et al. 2008). One possible explanation for why mating barriers are much stronger in *L. saxatilis* than in *L. fabalis* may be that the size difference between the ecotypes are more pronounced in *L. saxatilis* than in *L. fabalis*. In the former species the larger ecotype is on average at least twice the size of the smaller ecotype

(Sundberg 1988), while in *L. fabalis* the females of the larger ecotype are, on average, only about 43 % larger than the males of the smaller ecotype (Saltin unpublished data). Previous studies of *L. littorea* using similar size difference between size classes, larger snails being 40-50% larger than the small ones, also showed that males preferred large females (Erlandsson and Johannesson 1994). Although males of littorinids in general tend to preferentially mate with larger females, as these are more fecund than small females (Hughes and Answer 1982; Janson 1985; Erlandsson and Johannesson 1994; Dewitt 1996; Yusa 1996; Angeloni 2003), it seems quite likely that this effect will only persist up to a certain size difference, and with much larger females, males will possibly no longer be able to mate these, due to mechanical difficulties during copulation. Even though evidence for size-based mechanical barriers in nature are scarce, Richmond and co-workers (2011) found that assortative mating in a lizard species complex can largely be explained by a mechanical barrier caused by size differences. Thus, in littorinid snails it is possible that male preference for large females might level off at some point when size differences become too large, and consequently, beyond this point male tracking and copulation of females would no longer be favoured by selection and hence less frequent. Overall, this should result in a hump-backed relationship between mates' size differences and males' willingness to mate (Fig. 4).

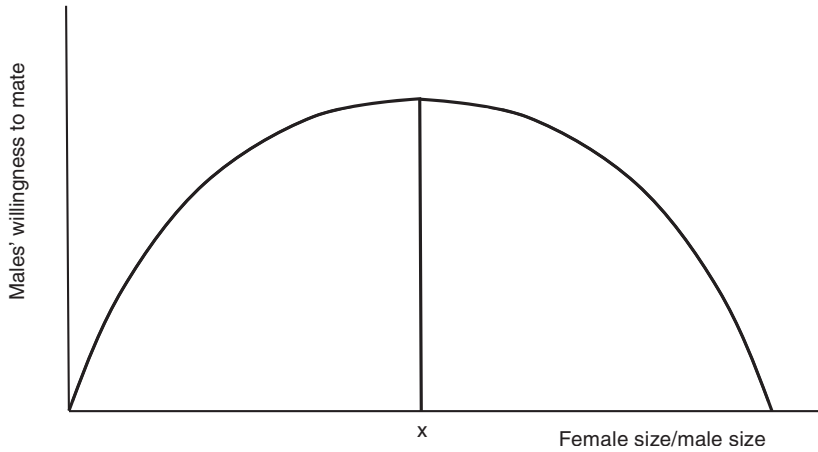


Figure 4. Hypothetical model over males' willingness to mate with females. Males are expected to show increasing interest in females up to a point (x) where the female is relatively larger than the male and after that point, the interest should decrease due to mechanical difficulties when a male try to mate a too large female.

Size differences evolve during local adaptation of snail ecotypes to different microhabitats, but as the comparison between *L. fabalis* and *L. saxatilis* shows, the size relationship between male and female may be critical to how size differences affect gene flow between ecotypes. One consistent result over three different species of *Littorina* (*L. fabalis*, *L. saxatilis* and *L. littorea*) is that larger males express negative mating preference towards smaller females. In both *L. fabalis* and *L. saxatilis* this imposes a partial barrier to gene flow between ecotypes of the same species. But while the barrier is unidirectional in *L. fabalis* and only hinders gene flow between LM males and SS females there is (as described earlier) also a barrier in the other direction in *L. saxatilis* where ecotypes differ substantially in size, resulting in a strongly impeded gene flow between the two ecotypes. Thus, the magnitude of the size difference between males and females might affect male willingness to mate, in one or the other direction; with implication on the evolution of reproductive barriers, ecotypes and eventually species.

Reproductive barriers and conflicts in mate recognition

Reproductive barriers are often in focus in speciation research since isolating barriers between groups are central to the Biological Species Concept (Mayr 1942;

Mayr 1995), and thus the evolution of these are believed to be at the very heart of species formation. Reproductive barriers can be divided into three categories: (1) premating isolating barriers, that counteract gene flow before transfer of sperm or pollen, (2) postmating, prezygotic isolating barriers that operate after sperm or pollen transfer but hinders fertilization and (3) postzygotic isolating barriers through hybrid inviability or sterility (Coyne and Orr 2004). Premating isolating barriers are often considered to be more important during early phases of speciation, e.g. behavioural isolation that counteract heterospecific sexual interaction (Coyne and Orr 2004), however, as shown below, some species of *Littorina* provide notable exceptions.

Mate recognition, including recognition of individuals of the right sex and species and recognition of high quality mates, is important for all sexually reproducing organisms (Pfenning 1998). However, species recognition and mate-quality recognition can interact either by reinforcing each other, resulting in diversification of sexually selected traits among closely related species (Andersson 1994), or by opposing each other, in situations where high-quality conspecifics resembles heterospecifics (Pfenning 1998). This conflict between species- and mate-quality recognition may result in seemingly maladaptive heterospecific sexual interactions (Pfenning 1998).

In **Paper III** we studied male mate choice, during mate search, mounting and mating, in the sister-species *L. fabalis* and *L. obtusata*. We found that there are premating isolating barriers between the species acting during direct contact at the stage of initiation of copulation, although *L. fabalis* males seem to be unable to differ between species from mucus trails. On the contrary, males rather followed females of *L. obtusata* than conspecific females (Fig. 5A). This could be explained by males' preference for large females (Rolán-Alvarez, Zapata et al. 1995; **Paper II**), since *L. obtusata* is larger than *L. fabalis*. This seemingly maladaptive behaviour could be explained by a conflict between species- and mate-quality recognition, where the trails of the most desired conspecific females resembles the trails of heterospecific females. Even though males seem unable to discriminate between species from mucus trails they could do so in close contact with females. That is, we found that *L. fabalis* males initiated mating with *L. obtusata* females less often compared with conspecific females and heterospecific copulation attempts were also interrupted after just a few minutes (Fig. 5B and Fig. 2 in **Paper III**).

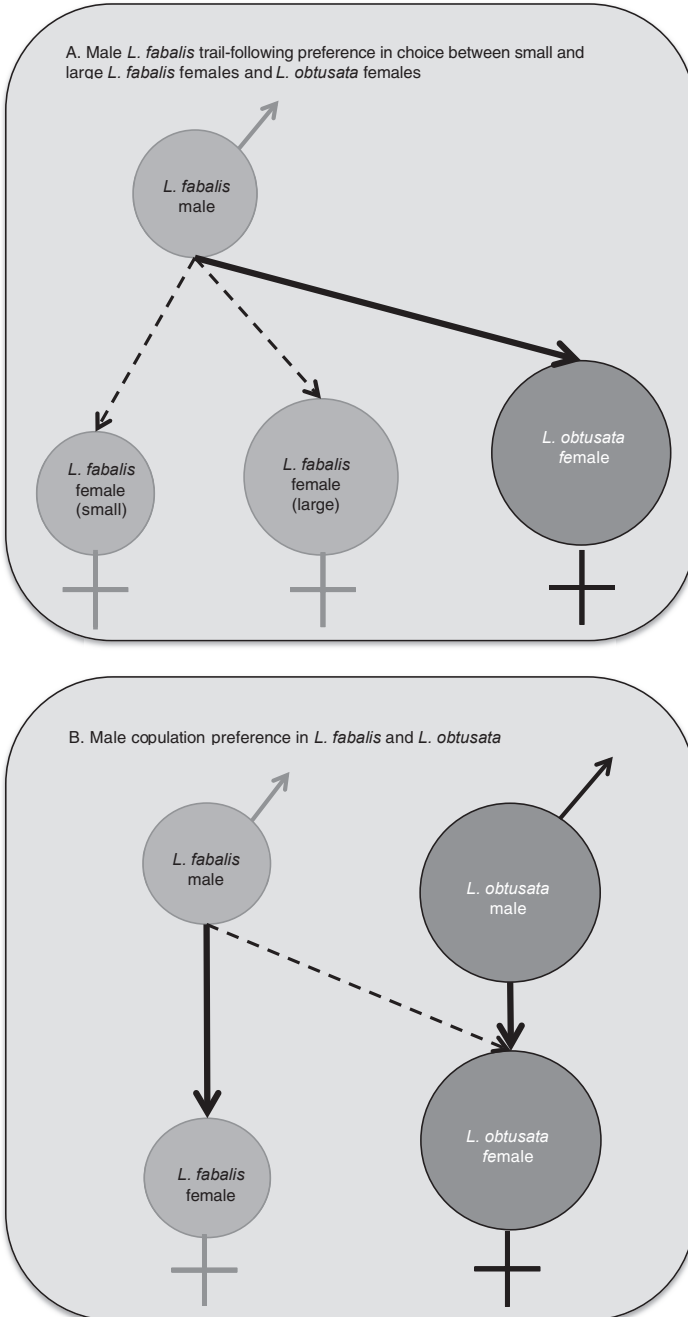


Figure 5. Mate preference in male *L. fabalis* and *L. obtusata* during (A) trail-following and (B) copulation. Preference is indicated by the thickness of arrows: thick arrows indicate significant preference for one group of females.

Although the mechanism of species recognition during close contact remains unknown, it has been observed that males move in a stereotypic way after mounting a female (Saur 1990), which might be a way for males to evaluate their partner and prohibit interspecific copulations. Since we also found that interspecific copulations were interrupted, it is likely that there are species recognition mechanisms acting during or at the initiation of copulation. One of the most pronounced differences between the species, and the only diagnostic morphological character, is the dissimilarity in male genital morphology; with differences in arrangement of mamilliform penial glands and also pronounced differences in penial filament, which is much longer in *L. fabalis* compared to *L. obtusata* (Reid 1996; Fig. 6). There are also differences in female genital morphology as *L. fabalis* have a much shorter bursa copulatrix compared to *L. obtusata* (Reid 1990). Although it remains to be tested, it is possible that the dissimilarity in genital morphology is involved in species recognition. Moreover, a recent study of divergence in penis morphology among sister-species pairs of littorinids show that there is significantly more divergence among sympatric sister pairs, indicating that the differences might have evolved to reinforce prezygotic mating barriers between close species (Hollander, Smadja et al. 2013).

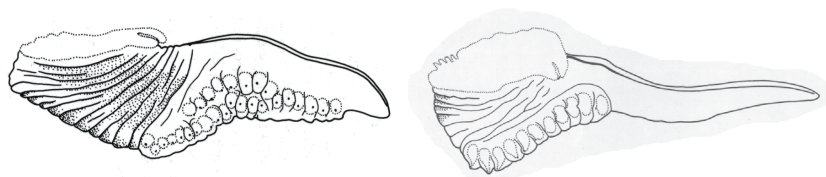


Figure 6. Male genitalia in *L. obtusata* (to the left) and *L. fabalis* (to the right). Source: Reid 1996.

Our results indicate that *L. fabalis* males assess female quality extremely early, already during trail-following, even though there is a risk of taking the wrong decision with respect to species at this stage. Possibly males can afford to take this risk if the rewards for mating large conspecific females are high and if mistakes occur rarely due to vertical separation of the two species on the shore (Williams 1990). In Sweden, however, the overlap is wide compared to other parts of Europe due to narrow tidal range and heterospecific mating attempts do occur in the field (Saltin, pers. obs.). Therefore, it is surprising that there seems not to be any species specific

cues in the mucus trails, so that males can avoid following heterospecific females. Possibly the evolution of a species recognition system, including signalling and detection by receptors, is complicated and might not have had time enough to evolve yet. Indeed, the Swedish populations are relatively young as the shores must have been colonized by snails after the end of the last glaciation approximately 15,000 - 13,000 years ago (Eliasson, Lundqvist et al. 2003).

Postcopulatory sexual selection

Polyandry is common among animals and it has been shown in many taxa that multiple mating can be beneficial for females, e.g. by increasing the reproductive output or the genetic variation of the offspring (Jennions and Petrie 2000; Simmons 2005). In females that have mated with several males, there is potential for postcopulatory sexual selection (Birkhead and Moller 1998). This can be male driven by sperm competition, giving the male with the most abundant or most competitive sperm, or the first or last male, a greater chance to fertilize more eggs than average (Birkhead and Moller 1998). It can also be female driven by cryptic female choice, if females can influence the process of fertilization after copulation (Eberhard 1996). In **Paper IV** we examined paternity distribution, using genetic tools, among broods of females of *L. saxatilis* that had been mating with one, two, five and ten males in laboratory. Multiple paternity was common in families where the female had mated two or more males and the distribution of fathers in those families was not random. There were more males than expected by chance that fathered very few offspring and one or a few males in each family that sired a majority of the brood, a pattern that would not be expected from random fertilization. This indicates that there is postcopulatory sexual selection influencing fertilization; either by sperm competition or cryptic female choice.

We also analysed offspring production and found that the number of males that the females had been mating with was not influencing offspring production. This raised the question why females of this species are extremely promiscuous with field data showing that females carry offspring sired by up to 20 males (Panova, Boström et al. 2010), when mating with only one male seem to be enough to ensure fertilization (**Paper IV**). Indeed, since there are costs of mating (**Paper V**) there should be fitness benefits for multiple matings to outweigh these costs, if females should allow

additional matings. For example, having multiple fathers to the clutch will increase the genetic diversity among the offspring, which could be important in heterogeneous environments (Crean and Marshall 2009). Although genetic benefits have been proposed as an explanation for female promiscuity, modelling and empirical data challenge this hypothesis since several studies show that genetic benefits of multiple paternity is often marginal (Yasui 1998; Tronetti, Thurin et al. 2007; DiBattista, Feldheim et al. 2008). It could however be beneficial by increasing genetic variation during population bottlenecks such as re-colonisations after local extinctions, which have occurred during recent history of *L. saxatilis* (Johannesson and Johannesson 1995). Modelling shows that multiple paternity in *L. saxatilis* during a colonization event where only one mated female arrives to an empty site increases heterozygosity significantly (Rafajlovic, Eriksson et al. in prep.). However, since matings are costly for females, it is hard to explain how the benefits at the population-level may cause selection for high polyandry at the level of individual females. Instead, the most likely explanation is that females allow additional matings out of convenience, and accept the costs of superfluous copulations rather than take the costs of rejecting males (Thornhill and Alcock 1983; Panova, Boström et al. 2010). Postcopulatory sexual selection in combination with convenience polyandry is not unlikely as convenience polyandry often results in high levels of promiscuity and hence, increases the potential for sperm competition or cryptic female choice (Birkhead and Pizzari 2002). In cases where females mate out of convenience, cryptic female choice can also be a way for females to “take control” over fertilization when deprived of precopulatory choices (Thornhill and Alcock 1983; Thiel and Hinojosa 2003; Brennan, Prum et al. 2007).

Sexual conflicts

Various types of sexual conflicts are found in nature, and a common type is a conflict between males and females if the number of matings that maximize the individuals' fitness differs between the sexes (Arnqvist and Rove 2005). The basis for sexual conflicts originates from males having numerous and small gametes whereas females have few and large gametes. Hence females invest more in each offspring than do males on the gamete level (Bateman 1948). Consequently, males generally have the potential ability to produce many more offspring than females (Parker and

Simmons 1996). In species with no paternal care, mating repeatedly is the only way for males to increase their reproductive fitness, while females having received enough sperm to fertilize all the eggs may not benefit from further matings (Arnqvist and Nilsson 2000). Often, however, females can increase their fitness by receiving additional sperm to increase genetic diversity among the offspring, to trade up for a higher quality mate, or to reduce risk of only mating with infertile or incompatible mates (Jennions and Petrie 2000). There are also other more specific cases where females receive, for example, a nutrition resource by additional matings (Boggs 1995), or where sexual contact is important for social interactions (Savage-Rumbaugh and Wilkerson 1978). But often the costs of mating exceed the benefits at a lower mating frequency for females than for males resulting in a sexual conflict over mating frequency (Fig 7).

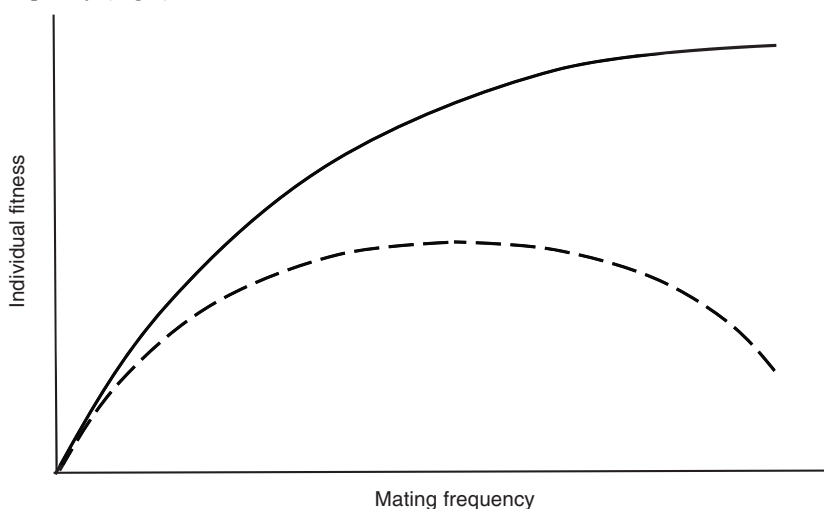


Figure 7. Generally individual fitness for females (dashed line) start to decrease at lower mating frequency compared to males (solid line). Source: Drawn from an idea presented in Arnqvist and Rove (2005).

In **Paper V** we argue that the inconsistent pattern of mating behaviour we observed in four species of the genus *Littorina* can be explained by an underlying sexual conflict over mating frequency between the sexes in one of the species. In three of the four Swedish species of *Littorina* (*L. fabalis*, *L. obtusata* and *L. littorea*) males have the ability to determine if a trail belongs to a male or a female and follow trails from females longer than males (Fig. 8A). How this is done is still unknown, but

the most likely explanation is that there is a gender specific chemical in the mucus that males' can detect. However, in the fourth species, *L. saxatilis*, males were following conspecific trails randomly with respect to the gender of the marker snail (Fig. 8B), indicating that males of this species were unable to distinguish between male and female trails. It is likely that the common ancestor to all four species had the ability to detect gender from mucus trails, and that this ability was lost in *L. saxatilis*. This is surprising since being able to rule out half of the trails encountered and only follow female trails seems to be highly adaptive for males. The explanation to this seemingly strange pattern lies in the life-history of these species (Table 1) and in a sexual conflict. The three species that have the ability to detect females (*L. fabalis*, *L. obtusata* and *L. littorea*) have higher or much higher egg production and shorter seasons of mating than *L. saxatilis*, and most importantly, they also live at much lower population densities than does *L. saxatilis*. The differences in life-history contribute to make females of *L. saxatilis* the least sperm-limited of the four species. Indeed, *L. saxatilis* have been found to be highly promiscuous, more so than, for example, *L. obtusata* (Paterson, Partridge et al. 2001; Mäkinen, Panova et al. 2007; Panova, Boström et al. 2010). The total mating cost is probably much higher in *L. saxatilis* than in the other three species with their lower population densities (hence lower mate encounter rate), and to reduce this cost selection has favoured females of *L. saxatilis* that do not reveal the sex in the mucus trail. This gives rise to a sexual conflict within *L. saxatilis* where males try to mate as much as possible and females try to avoid male harassment by disguising their gender. Further strength for this conclusion was provided when we found that *L. saxatilis* males could indeed distinguish between mucus-trails of male and female *L. fabalis* (Fig. 8C). The females of this species leave a “female mark” in their trails that male *L. saxatilis* still recognise and follow, showing that males (if they have the chance) follow “female-marked” trails.

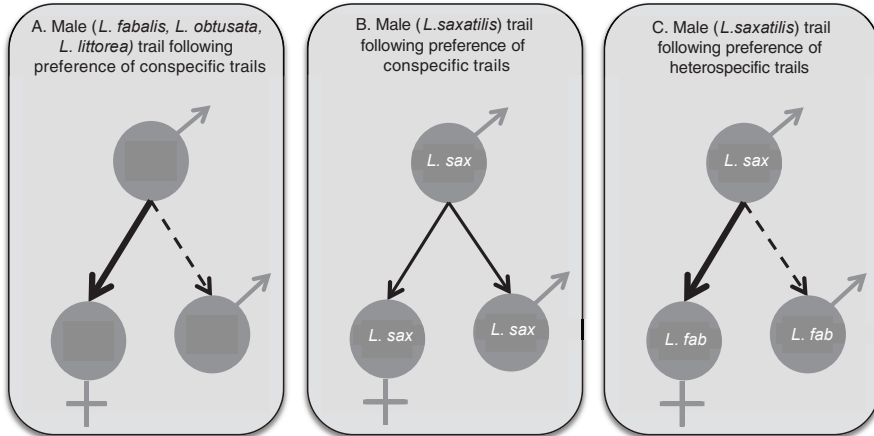


Figure 8. Male trail-following preference for conspecific males and females in (A) *L. fabalis*, *L. obtusata* and *L. littorea* and in (B) *L. saxatilis*. Male *L. saxatilis* preference for heterospecific (*L. fabalis*) male and female trails is shown in (C). Preference is indicated by the thickness of arrows: thick arrows indicate significant preference for one group.

Female trail-masking may also explain earlier surprising observations that *L. saxatilis* often engage in male-male copulations (Saur 1990; Erlandsson 2002), as males of this species follow any trail encountered and probably also initiate copulation with any conspecific snail before being able to separate between male and female mates.

There is a similar case of gender mimicry as a result of a sexual conflict over mating frequency in damselflies, where females mimic males in colour and behaviour to reduce number of matings and harassment during mating (Andres, Sanchez-Guillen et al. 2002). And there are other similar examples among insects and lizards (Galan 2000; Reinhardt, Harney et al. 2007), but **Paper V** provides us with the first example in a marine invertebrate species.

CONCLUSIONS

Although mucus production primarily has evolved to facilitate locomotion, there are many secondary functions of trail-following and several of these have high relevance to our understanding of the evolution of mating behaviours, reproductive barriers and speciation (**Paper I**). In addition to trail-following, there are at least four themes that emerge as central to our understanding of snail mate choice and its evolutionary consequences (Fig. 9):

Size is a crucial trait in male mate choice of *Littorina*, both during trail following and copulation (**Papers II and III**). While a strong preference for large females might have evolved to increase male inclusive fitness (by mating a more fecund female), size seems to be involved in partial reproductive barriers, and may eventually be a proximate driver of speciation. Hence, sexual selection on size seems a very important component of snail evolution. In addition, postcopulatory sexual selection has been proposed to be important in gastropods (Chase 2002). The observed pattern of non-random paternity found in *L. saxatilis* (**Paper IV**) indicates that there is postcopulatory sexual selection in this species, although more studies are needed to unravel its causes and consequences. Postcopulatory sexual selection is partly a consequence of the extreme promiscuity that is found in *L. saxatilis*, a phenomenon that also gives rise to a sexual conflict over mating frequency (**Paper V**). Another conflict may explain the seemingly maladaptive behaviour of heterospecific sexual interactions (**Paper III**). This conflict being between optimizing species recognition and high-quality mate recognition among males. Thus, in many respects, snail behaviours are tightly linked to the evolution of these species, and not least to the evolution of reproductive barriers. As members of the genus *Littorina* are popular models for studies of the formation of reproductive barriers, ecotypes and species, insights about mate choice and its effects on reproductive barriers, is highly relevant for our understanding of the evolution of new species. Specifically, the four steps recognized in the mate choice of *Littorina*; trail-following, mounting, copulation and postcopulatory mate choice (Fig. 2), provides a useful framework for further research on aspects of mate choice and its evolutionary and ecological implications.

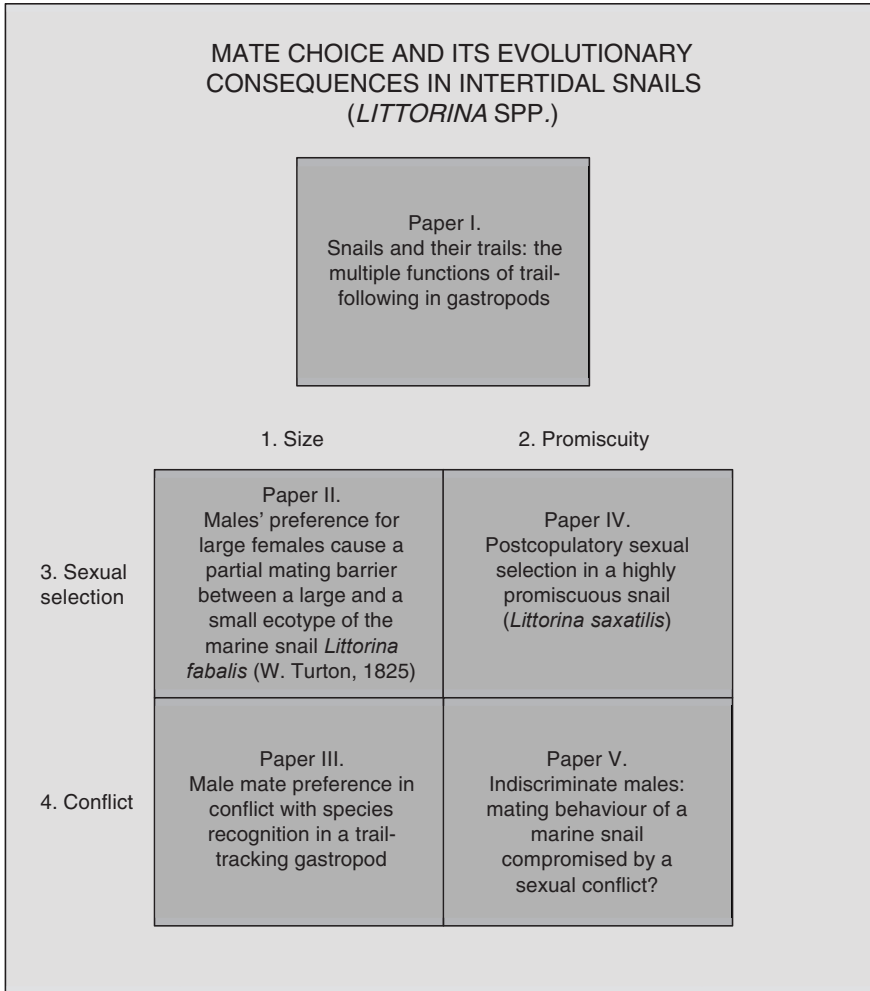


Figure 9. The content of this thesis, the interlinked themes (1-4) within it and the papers where these themes appear.

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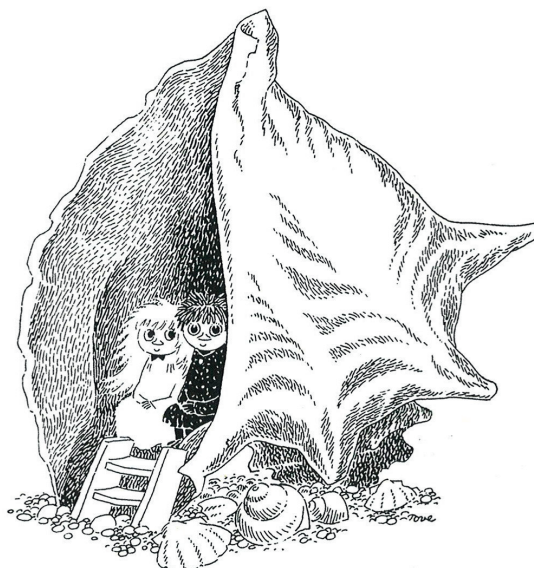
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*... och sen
levde de lyckliga
i alla sina dagar...*

SLUT

Picture: T. Jansson (1960).