Sexual selection in a simultaneous hermaphrodite: mate choice and sperm utilization patterns

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SUMMARY

Sexual selection processes, in general, are distinguished in precopulatory and postcopulatory mechanisms. In the animal kingdom, precopulatory mechanisms are related to mate competition and mate choice before the copulation. Postcopulatory mechanisms like sperm competition and cryptic female choice occur inside the female reproductive organs and can influence the reproductive outcome due to differences in sperm utilization for the fertilization of the ova. In simultaneous hermaphrodites, both sexes are present in an individual, therefore sexual selection processes are influenced by the interests of both genders.

In the present thesis, I combined different approaches including behavioural studies, evaluations of life-history traits and genetic parentage analyses to broaden our view on the sexual selection, particularly in mate choice and sperm utilization patterns, in the model organism *Arianta arbustorum,* a simultaneously hermaphroditic land snail. Furthermore, a novel immunocytochemical approach was developed to allow further studies relating to postcopulatory selection mechanisms in the female reproductive organs.

In order to assess derminants of mating success and female and male reproductive success in individuals of *A. arbustorum*, we video-recorded the behaviour of individually-tagged snails kept in groups of six animals over one reproductive period and assigned the genotyped hatchlings to the female and male function of individual parents. Mating success, which is equal to the female and male function in simultaneous hermaphrodites with reciprocal copulation, was mainly determined by the activity of an individual. We found that female reproductive was positively correlated with male reproductive success and that both were determined by the individual's activity. These results contrast the existing sex allocation theory.

In order to assess mating frequency and the sperm utilization patterns in the wild, microsatellite DNA markers were applied to reconstruct the patterns of paternity of 1088 offspring from 26 mother snails that copulated in four natural populations. Overall, multiple paternity was detected in every mother-progeny array ranging from two to six fathers. Moreover, depending on the population density, the four populations examined differed in the level of multiple paternity. The results revealed also highly skewed paternity patterns in the progeny of 57.7 % of the mother snails, *i.e.* the number

and identity of fathers siring the offspring of single mothers also varied among successive clutches. Furthermore, genetic analyses indicate a low level of self-fertilization in one of the four populations.

In species with multiple mating and long-term sperm storage males are expected to show a preference for mating with virgin and young females to reduce the risk of sperm competition. To examine whether sperm transfer to and sperm utilization of virgin mating partner are postcopulatory preferred over those of an experienced mate, polymorphic microsatellite markers were applied to assess sperm utilization and last mate sperm precedence in hatchlings of copulating pairs from the wild consisting either of two adults, two subadults or of one adult and one subadult snail. The results showed that adult snails used sperm received from subadult mating partners for egg fertilization in the same frequency as sperm from adult partners, indicating that subadult and adult snails do not differ in male function. Furthermore, in 35% of the subadult individuals sperm stored from previous mating(s) was utilized for the fertilization of the ova. However, compared with adults, these young individuals exhibited a lower risk of sperm competition to their partners, indicated by a higher last mate sperm precedence. On the other hand, subadult snails produced fewer eggs than adult snails, which may counteract the evolutionary advantage of preferring a young partner with low sperm competition risk.

Sexual selection goes hand-in-hand with reproductive isolation and both can influence gene flow, genetic diversity and biodiversity. Different mechanisms of reproductive isolation and sexual selection were examined in individuals of *A. arbustorum*. Snails from two geographically isolated populations in the Swiss Alps were allowed to copulate with both a homotypic (individual from the same population) and a heterotypic (individual from the other population) partner (in half of the pairs in reversed order). In the first mating, successful copulations occurred in a lower frequency in heterotypic pairs (55.6%) than in homotypic pairs (82.9%). Heterotypic pairs that eventually copulated showed more breaks during courtship than homotypic pairs. However, neither the number of eggs produced nor their hatching success was influenced by the type of mating partner. In the second mating, the sequence of different partners had an effect on the proportion of successful copulations in snails from one geographical population. Paternity analyses of progeny of snails that mated twice indicate no influence of the origin of mating partner. These findings indicate the presence of partial

precopulatory isolation between two distant snail populations, although reproductive compatibility is still maintained.

To understand the patterns of sexual selection after copulation, the mechanism of sperm storage, sperm utilization and sperm digestion are of relevant importance. A novel immunocytochemical technique to track the fate of labelled sperm in the female reproductive organs of invertebrate species was developed and tested on individuals of *A. arbustorum*. The proportion of sperm labelled among the sperm produced by individuals averaged 99.3% and labelled sperm could be reliably visualized in both the sperm storage and sperm digestion organs of all receivers examined. The novel technique presented can be easily adjusted to other invertebrate species. This allows the study of mechanisms underlying postcopulatory sexual selection, and thus it improves our understanding of the evolution of male and female reproductive morphology and physiology in a variety of species. Moreover, in double-mated individuals the sperm-labelling technique can provide insight into the mechanisms underlying sperm competition and cryptic female choice.

GENERAL INTRODUCTION

In the recent years, the diversity of biological organisms in natural environments has become a very important issue in scientific research. Biodiversity includes not only the variety of species; also diversity of ecosystems and genetic diversity are interwoven with the term of biodiversity. Charles Darwin (1809–1882) published in his books "The origin of species" (1859) and "The descent of man, and selection in relation to sex" (1874) the source ideas on how natural forces, *i.e.* natural and/or sexual selection, can drive processes of evolution and speciation to the today's existing biodiversity. Commonly, selection favours traits with positive effects in species survival in the occupying ecosystem. Consequently, new traits can guide to the establishment of a new species. Such evolutionary shifts in a species seldom arise spontaneously, but often gradually over generations (Barton and Charlesworth 1984). Moreover, during speciation the development of reproductive preferences can occur, i.e. individuals with new traits prefer mates with the same traits over mates exhibiting "ancient" traits. So, sexual selection and reproductive isolation go hand-in-hand and both influence speciation and biodiversity (Coyne and Orr 2004, Rundle and Nosil 2005, Ritchie 2007).

However, mate and sexual selection aim not only at differences in morphological characters, but also at differences in genetic and fitness traits (Andersson 1994). The benefit in evolving sexual selection strategies arises in gaining the most effective reproductive success, *i.e.* a high contribution of fit offspring to the species population assets. Hence, sexual selection mechanisms are widespread evolved and divers in animal, but also in plant species (Andersson 1994, Grant 1995). In general, sexual selection is divided into processes that occur before mating (= precopulatory selection) and inside the female organs after the mating (= postcopulatory selection). Precopulatory mechanisms include male competition and female mate choice (Birkhead amd Møller 1998). Postcopulatory sexual selection can either be caused by sperm competition between different mating partners or by cryptic female choice and selective sperm utilization (Birkhead and Møller 1998, Birkhead and Pizzari 2002). Sperm competition arises when fertile sperm from two or more males co-occur within a female reproductive tract and compete to fertilize her ova (Parker 1970). The resulting evolutionary arms race has led to a variety of behavioural, morphological and

physiological adaptations which either enhance the competitive advantage of a male's sperm or counter the sperm of competitors (Birkhead and Møller 1998, Birkhead *et al*. 2009). However, females are also able to influence paternity contribution of co-occurring sperm from different males in their reproductive organs. Hence, sperm of different competing donors can be differently utilized for the fertilization of the ova due to cryptic female choice (Eberhard 1996). Because of the simultaneous presence of sperm competition and cryptic female choice hidden inside the female reproductive organs, these postcopulatory processes are difficult to observe and difficult to clearly partition. However, they are playing an important role in sexual selection and therefore, research dealing with postcopulatory sexual selection has become a relevant issue in the last years.

In gonochoristic animals, divers processes concerning precopulatory and postcopulatory sexual selection are described in a variety of species (Andersson 1994, Arnqvist and Rowe 2005). In contrast, in simultaneous hermaphroditic species, where both genders are present at the same time, studies dealing with precopulatory and postcopulatory sexual selection are rare.

Interestingly precopulatory sexual selection processes are influenced by both the female and the male traits of an individual in simultaneously hermaphroditic animals. That means, during simultaneous intromission and sperm exchange a sperm donor is at the same time a sperm receiver. Thus, a sexual conflict between female and male interests may arise within an individual (Michiels 1998). Therefore, Greeff and Michiels (1999) suggested that precopulatory selection on traits related to mate acquisition is intrinsically weaker in hermaphrodites than in gonochorists and that the postcopulatory mechanism might be more essential. Additionally, in several hermaphroditic species, long-time sperm storage from different mating partners, complex sperm storage organs and a mechanism for the digestion of excess sperm are known (Baur 1998, Beese *et al*. 2006, Beese *et al*. 2009) and can influence the outcome of sperm utilization (Haase and Baur 1995, Chase and Darbyson 2008).

Hermaphrodites have, unlike gonochoristic species, the potential to utilize their own sperm to fertilize their ova, which is determined as self-fertilization. It is assumed that the ability to reproduce by self-fertilization is a means of coping with limited dispersal ability or low mate encounter rate in low-density populations (Levins 1968). Mixed mating systems, where reproduction occurs via self- and cross-fertilization, are common

in plants (Goodwillie *et al*. 2005) and have also been found in a variety of simultaneously hermaphroditic gastropods (Baur 1987, Heller 1993, Jarne and Auld 2006). In general, complete outcrossing is preferred when the inbreeding effects are pronounced (Lande and Schemske 1985). Like inbreeding (*i.e*. mating between close relatives) and outbreeding (*i.e*. reproduction between unrelated individuals from different populations (heterotypic)), self-fertilization reduces genetic diversity and fitness in natural populations (Frankham 1995, Auld and Relyea 2010). However, in natural populations it is difficult to clarify the occurrence of self-fertilization.

While self-fertilization, inbreeding and outbreeding can negatively influence genetic diversity and fitness traits in a population, mating frequency can positively influence the genetic diversity and therefore, the fitness of individuals in a population (Reed and Frankham 2003). In gonochoristic species, male's mate acquisition is frequently the most severe limitation on reproductive success and, consequently, male strategies to ensure mating with multiple females are common and widespread in virtually all animal taxa (Simmons 2001, Uller and Olsson 2008). In contrast, female reproductive output in terms of the number of offspring is commonly not limited by the number of mating partners. Therefore, selection on multiple mating in females should be weaker than in males, especially in species with internal fertilization. In simultaneous hermaphrodites, mating strategies have to deal with the interests of both genders in an individual and the allocation of resources in one or the other gender part (sex allocation; West 2009). Nevertheless, simultaneous hermaphrodites are affected by forces similar to those leading to complicated mating strategies and sperm competition in animals with separate sexes (Charnov 1996, Michiels 1998, Anthes *et al.* 2006). Due to the notorious difficulty with which mating can be reliably observed in wild populations, research on mating strategies in hermaphrodites has been hampered and is rarely documented in the literature.

In the last decade, the development of molecular techniques and software programs became a keystone in the investigation of several biodiversity aspects and topics of sexual selection (Etges and Noor 2002). Therefore, promising genetic tools can now support the investigation of self-fertilization rate or mating frequencies in the wild (Johns and Arden 2003, Jarne and David 2008), especially in invertebrates with a rather cryptic life.

FOCUS OF THE THESIS

The aim of my thesis was to improve our understanding of sexual selection mechanisms in simultaneous hermaphrodites. As model organism, we considered the simultaneously hermaphroditic land snail *Arianta arbustorum*. In particular, we used the results of behavioural studies, findings in life history traits and genetic analyses to broaden our view on mate choice and sperm utilization patterns in *A. arbustorum*. Furthermore, a novel immunocytochemical approach was developed to allow further studies about mechanisms underlying sexual selection and sperm utilization patterns inside the female reproductive organs.

In **Chapter 1**, we considered behavioural and genetic traits to assess mate choice and patterns of sperm utilization as well as the factors involved with the female and male reproductive success in individuals of *A. arbustorum*. We kept snails in groups under semi-natural conditions and video-recorded their behaviour and the mating frequency over a reproductive season. Furthermore, we genotyped emerged hatchlings and their potential parents to allow a kinship reconstruction. The genetic diversity of the parents was assessed to examine any potential influence on the reproductive success.

Mating frequency has important implications for patterns of sexual selection and sexual conflict and hence for issues such as the maintenance of genetic diversity and speciation. In **Chapter 2** we examined mating frequencies, sperm utilization patterns and self-fertilization in offsprings of individuals from natural populations of the simultaneous hermaphrodite land snail *A. arbustorum*. We assessed the level of multiple paternity and sperm utilization patterns using polymorphic genetic markers.

In species with multiple mating and long-term sperm storage, males are expected to show a preference for mating with virgin and young females to reduce the risk of sperm competition. In **Chapter 3,** patterns of postcopulatory sexual selection in respect to sperm utilization, in particular the potential discrimination of sperm of subadult or fully-grown (adult) partners, was examined. For this study, we collected copulating pairs consisting either of two adults, two subadults or of one adult and one subadult snail in the wild and determined the paternity of their hatchlings that emerged from subsequently deposited eggs.

Reproductive isolation is an important step in the process of speciation. The occurrence of reproductive isolation mechanisms between natural populations has an

essential impact on the reduction of gene flow and can therefore influence the genetic diversity and fitness of populations. In **Chapter 4** we examined different mechanisms of reproductive isolation in the simultaneously hermaphroditic land snail *A. arbustorum*. We conducted mating trials with individuals from two geographically isolated populations to obtain combinations of double-mated snails. We evaluated mating behaviour and paternity contribution in the produced offspring to gain insight in the impact of reproductive isolation and sexual selection occurred.

In **Chapter 5**, we present a novel immunocytochemical sperm-labelling technique to track the fate of BrdU-labelled sperm in the female reproductive organs of invertebrate species. We tested this technique by assessing the sperm labelling success and the reliability of detecting labelled sperm in the spermatheca (sperm storage organ) and bursa copulatrix (sperm digesting organ) of receiver individuals of *A. arbustorum*.

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CHAPTER 1

Determinants of female and male reproductive success in a simultaneous hermaphrodite land snail

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Classical sexual selection theory assumes that the reproductive success of females is limited by the resources available for egg production, while the reproductive success of males is determined by the number of mates (Bateman's principle). It has been suggested that the optimal mating rates should also diverge between gender functions within individuals of simultaneous hermaphrodites. We assessed determinants of mating success and female and male reproductive success in individuals of the simultaneous hermaphrodite land snail *Arianta arbustorum*. We videorecorded the behaviour of individually tagged snails kept in groups of six animals over one reproductive period (58 days) and assigned the genotyped hatchlings to the female and male function of individual parents. We found considerable interindividual variation in the activity of snails, which is a combined measure of time spent crawling, feeding and digging. The snails mated between zero and three times. Mating success, which is equal to the female and male function in simultaneous hermaphrodites with reciprocal copulation, was mainly determined by the activity of an individual. We found that female reproductive success (number of hatchlings emerging from the eggs laid by the focal snail) was positively correlated with male reproductive success (number of hatchlings sired by the focal snail) and that both were determined by the individual's activity. Furthermore, both female and male reproductive success of an individual were influenced positively by the snail's degree of genetic heterozygosity and negatively by shell size. Our results challenge the trade-off assumption of sex allocation theory in simultaneous hermaphrodites.

Much recent research effort has been directed at explaining determinants of the reproductive success of females and males in gonochoristic animals (Clutton-Brock 1988; Roff 2002). In mating systems without paternal care, male fitness tends to be more tightly linked to mating success than is female fitness (Trivers 1972). This can be explained by Bateman's principle, which states that the female's reproductive success is primarily limited by the energy available for producing gametes, whereas the reproductive output of males is primarily governed by the number of mates (Bateman 1948). However, in species in which individuals are promiscuous, sexual selection continues after copulation in the forms of sperm competition and female manipulation of sperm. Sperm competition occurs when spermatozoa from different males compete in the reproductive tract of a female for the fertilization of her eggs (Parker 1970). In a variety of species, females have a physiologically and morphologically complex reproductive system, which may enable them to control or influence offspring paternity by post-copulatory sperm storage and selective sperm use (Eberhard 1996). There is increasing evidence that females choose between sperm from different males after copulation and that this so-called cryptic female choice may also affect paternity (Pitnick et al. 2009).

Simultaneous hermaphrodites are functional female and male at the same time. This type of gender expression is widespread in the animal kingdom and among plants (Michiels 1998; Jarne & Auld 2006). Bateman's principle also applies to hermaphrodites (Charnov 1979; Anthes et al. 2010). Despite the central role of number of mates in sexual selection theory, only very little is known of the absolute number of mating partners simultaneous hermaphrodites can acquire and how this number varies between individuals. For example, laboratory experiments with the hermaphrodite flatworm *Macrostomum lignano* revealed considerable variation in the number of mates and in sperm transfer success between individuals (Janicke & Schärer 2009). The number of matings increased with group size. However, food availability, and not group size, had a significant effect on female fecundity (Janicke et al. 2011). Furthermore, our knowledge of factors responsible for the variation in reproductive success via either sex function is still

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limited. The optimal mating rates may diverge between sex functions within an individual (Anthes et al. 2006). The possibility of a flexible division of resources between male and female functions as well as conflicts between individuals over sexual roles may further complicate sexual strategies in simultaneous hermaphrodites (Schärer 2009). It has been suggested that having both sexes combined in the same individual may limit sexual selection for traits involved in mate acquisition (Greeff & Michiels 1999). However, other features, such as multiple mating, sperm storage and internal fertilization, leading to traits for sperm competition and cryptic female choice, may be similar in simultaneous hermaphrodites and gonochoristic species (Baur 1998; Michiels 1998).

Research on mating strategies in simultaneous hermaphrodite land snails has been hampered by the notorious difficulty with which mating can be reliably observed in natural populations. Evidence for promiscuity and multiple paternity in broods is available for several snail species. Individuals of *Helix pomatia*, *Cornu aspersum*, *Cepaea nemoralis* and *Arianta arbustorum* have been observed to mate repeatedly with different partners in the course of a reproductive season resulting in multiple-sired broods (Wolda 1963; Baur 1988a, 1994a; Lind 1988; Rogers & Chase 2002; Evanno et al. 2005; Kupfernagel et al. 2010). However, little attention has been devoted to the fitness consequences of multiple mating in land snails (Jordaens et al. 2007).

We used a combination of behavioural and genetic data to examine the factors that influence female and male reproductive success in individuals of the simultaneous hermaphrodite land snail *A. arbustorum*. We kept animals in groups in a seminatural environment and videorecorded the behaviour of the individually tagged snails over one reproductive season. Using microsatellite markers, we genotyped the emerging hatchlings and assigned the offspring to the female and male functions of individual parents. We also examined the potential influence of heterozygosity of a parent snail on its female and male reproductive success because there is evidence in a variety of species that the fitness of an individual increases with increasing degree of heterozygosity (David 1998; Markert et al. 2004). As egg production is assumed to be more resource limited than sperm production in our model species, we predicted that male reproductive success would increase with increasing number of matings, while female reproductive success would level off with additional copulations. Furthermore, we asked which factors (behavioural traits, shell size, level of heterozygosity) determine mating and reproductive success in each sex function in individuals of *A. arbustorum*, and whether our results support existing models of sexual strategies in hermaphrodites.

METHODS

Study Species

Arianta arbustorum is common in moist habitats of northwestern and central Europe (Kerney & Cameron 1979). The snail has determinate growth (shell breadth of adults 17–22 mm). Individuals become sexually mature at 2–4 years, and adults live another 3–4 years (maximum 14 years; Baur & Raboud 1988). In the field, snails deposit one to three egg batches consisting of 20–50 eggs, per reproductive season (Baur 1990). Breeding experiments showed that 27% of virgin snails prevented from mating produced a few hatchlings by self-fertilization in the second and third years of isolation (Chen 1993). The reproductive success of selfing individuals, however, is less than 2% of that of mated snails, suggesting high costs for selfing (Chen 1994).

Mating in *A. arbustorum* includes elaborate courtship behaviour with optional dart shooting (i.e. the pushing of a calcareous dart into the mating partner's body), and lasts 2–8 h (Baur 1992). Copulation is reciprocal. After intromission, each snail simultaneously transfers one spermatophore (Haase & Baur 1995). The spermatophore is formed and filled with sperm during copulation (Hofmann 1923). It has a distinctive form consisting of a head, a body (sperm container with 800 000–4 000 000 spermatozoa) and a tail 2–3 cm long (Baur et al. 1998). Fertile sperm can be stored for more than 1 year (Baur 1988a). Mating is random with respect to shell size and different degrees of relatedness (Baur 1992; Baur & Baur 1997). Snails need at least 8 days to replenish their sperm reserves after a successful copulation (Locher & Baur 1999; Hänggi et al. 2002).

Paternity analyses in broods of wild-caught *A. arbustorum* revealed a high frequency of multiple inseminations (Baur 1994b; Kupfernagel et al. 2010). A controlled laboratory experiment showed that one successful copulation per reproductive season is sufficient to fertilize all the eggs produced by individual snails kept singly (Chen & Baur 1993). However, there is a probability of 5–8% that copulation will not lead to fertilization of eggs (no sperm transfer or transfer of infertile sperm; Chen & Baur 1993).

Experimental Animals

To obtain virgin snails we collected subadult individuals that had not yet completed shell growth from an embankment along a track in a subalpine forest near Gurnigelbad, 30 km south of Bern, Switzerland (46°45′N, 7°28′E) at an altitude of 1250 m above sea level. We kept the snails isolated in transparent beakers (8 cm deep, 6.5 cm in diameter) lined with moist soil (approximately 4 cm) at 19 °C and on a light:dark cycle of 16:8 h for 5 weeks. They were fed fresh lettuce ad libitum. During this period, subadult individuals reached sexual maturity as indicated by the formation of a flanged lip at the shell aperture. We marked the snails individually on their shells with symbols and lines drawn with correction fluid (Tipp-Ex). The animals showed no visible reaction to the marking procedure. We also measured the shell width of each snail to the nearest 0.1 mm using vernier callipers immediately before the experiment.

Video Tracking

We constructed observation chambers that allowed (1) continuous recording of snails over the entire reproductive period; (2) free movements of animals on natural substrate (soil); (3) egg deposition; (4) continuous identification of individual snails; and with (5) a seminatural temperature regime and a light:dark cycle of 16:8 h.

We kept groups of six randomly chosen snails in each observation chamber, a transparent plastic box measuring 29 × 19 cm and 17 cm high, lined with a 4 cm thick layer of moist soil and covered with a glass plate. Light was provided by cold-light sources (Osram Dulux L). A window kept constantly open allowed daily air temperature fluctuations. The room temperature varied from 16.5 to 24.1 °C (mean 22.1 °C). Fresh lettuce cut into small pieces was provided twice a week. Eight observation chambers with a total of 48 snails were arranged in a line.

To record the behaviour of the snails we used a computerized video-image technique. We installed a SONY camcorder with infrared illumination 80 cm perpendicular to each observation chamber. The eight cameras were connected via a Kramer VS-2081S 8x1 S-video switcher to a computer, which recorded a video frame from each camera at an interval of 2 min. As snails move slowly, this frame interval gave a reasonably accurate representation of the various behavioural elements. We recorded the snails' behaviour nonstop over a period of 58 days from 21 June to 18 August 2000. There were a few short gaps in recording owing to technical problems with the computer. Overall, data collected

over 95.4% of the experimental period of 58 days could be considered in the data analyses (see below). At night, when snails are mainly active, the records covered 96.0% of the period.

We exchanged the observation chambers every 3–4 days by transferring the animals to identical but clean chambers late in the afternoon before they became active. This procedure allowed us also to collect the eggs deposited in the soil with minimal disturbance of the experimental snails. We carefully examined the soil of the exchanged chambers for eggs, recorded the position of the batches (for a later determination of the mother), and collected the eggs. The eggs of each batch were incubated at 19 °C in petri dishes lined with damp paper towels. We counted newly hatched snails and removed them from remaining eggs to avoid egg cannibalism (Baur 1994c). Labelled in order of emergence, the hatchlings were stored at −80 °C until genetic analysis. We replaced snails that died during the study by new ones of equal size to maintain constant snail density. In the data analyses, however, we considered only the original snails that survived the entire experimental period.

Behaviour Analyses

Behavioural data are based on >300 000 video frames (>40 000 per observation chamber or individual snail). For all individuals we recorded six behaviours: (1) crawling: the head was moved forward and the tentacles were extended; (2) feeding: the snail was at the lettuce showing feeding movements; (3) digging: the snail was digging into the soil; (4) resting: the snail was stationary with its soft body retracted or entirely buried; (5) courtship: the snail had repeated oral contacts with a potential mating partner (usually accompanied by a slight eversion of the penial lobe) or was circling around the potential partner; and (6) copulation: simultaneous intromission of the penes of both mating partners. For each snail, the percentage of time spent performing each behaviour was calculated from the video frames using BioPictViewer version 1.0 (Zschokke 2002). We also determined the numbers of contacts a particular snail had with each of its five potential mates. We distinguished between short contacts (≤4 min, two snails in body contact on a single video frame or on two successive video frames) and long contacts (>4 min, body contact visible on three or more successive video frames). Short contacts were considered to be the result of random encounters of moving snails. In most cases, the snails went on their way ignoring each other. We did not consider short contacts in the analyses. However, we counted the long contacts for each snail. During long contacts, snails may assess the mating status, mating readiness or quality of potential partners. We defined mating success as the number of copulations a snail had.

We evaluated changes in the position of single snails on successive video frames using Big Sister version 1.2 (Zschokke 2003). The difference in a snail's position on two consecutive video frames represents the distance moved within 2 min. Summing these distances over 1 day or over 58 days results in the total distance moved per day or over the entire study period.

Paternity Analysis and Heterozygosity

DNA of individual hatchlings and parental snails (20–30 mg foot tissue) was extracted following a DNeasy protocol of Qiagen (2006). We screened the DNA of mother snails for microsatellite repeats using the nine primer pairs developed by Armbruster et al. (2005). Primers that did not amplify or produced only a small number of alleles were no longer considered. Six highly variable microsatellite loci (26, 55, A9, C3, H8 and H9) were employed for paternity analyses following the procedure described in Kupfernagel et al. (2010).

We genotyped 511 of 529 hatchlings from 37 mother snails. Hatchlings from one observation chamber were not genotyped because three mother snails died (see below). Null alleles, which can significantly affect estimates of genetic relatedness of individuals (Pemberton et al. 1995; Dakin & Avise 2004), were detected in six of the 511 hatchlings examined. Observed null alleles were a result of low DNA concentration and PCR failure. Data from these six hatchlings were omitted. Thus, in the paternity analysis, a total of 505 hatchlings were considered. This corresponds to 95.5% of all hatchlings that emerged from the eggs produced.

We considered the mother snails from the eight observation chambers as a reference sample of the 'population' ($N = 37$; the DNA of one snail could not be extracted; see below). The six loci used varied in the level of polymorphism from six to nine alleles. Genotype frequencies at all loci were within expectations of Hardy–Weinberg equilibrium ($P > 0.05$), and no evidence of genotypic disequilibrium between pairs of loci was found ($P > 0.05$). Paternity exclusion probability for all six loci combined was 0.94–0.99 (GenAlEx estimates; Peakall & Smouse 2006).

To estimate heterozygosity (H_o) of the parent snails, we calculated each individual's H_o by dividing the number of heterozygous loci by the number of genotyped loci. Our 'population' represents reasonable variation in H_o, with the 39 mother snails ranging in H_o from 0.33 to 1.00 on a scale of 0 to 1 (mean ± SE = 0.78 ± 0.03). We estimated genetic similarity as a pairwise relationship coefficient (Queller & Goodnight 1989) within copulating pairs of snails using SPAGeDi 1.0 (Hardy & Vekemans 2002).

Statistical Analyses

Eight of the 48 experimental snails (16.7%) died during the experiment. In the eight observation chambers, the number of snails that died were 0, 0, 0, 0, 1 (snail G1; see legend to Fig. 1 for abbreviations), 2 (D1 and D2), 2 (E3 and E6) and 3 (B3, B4 and B5). As explained above, dead snails were replaced by new ones to maintain density. However, we considered only the 40 original snails in the data analyses.

Microsatellite characteristics were assessed using both GenAlEx 6.1 (Peakall & Smouse 2006), an Excel add-on for population genetic data analyses, and GENEPOP (Raymond & Rousset 1995, http://genepop.curtin.edu.au). Sibship analysis and parentage reconstruction of the emerged hatchlings were performed with CERVUS 3.0 (Kalinowski et al. 2007). CERVUS uses multiple-locus data for likelihood assessment of parentage reconstruction in a population. The programme does not distinguish between maternal and paternal contribution. However, based on the video recordings, we could determine the mother of each egg batch. The maternal genotypes represent also the potential paternal genotypes in this simultaneous hermaphrodite. For the likelihood calculations, the mother snail genotypes of each observation chamber were used with the following input variables: simulation cycles = 50 000, probability of typed loci = 0.98, typing error = 0.02, confidence level = 95%. To check for paternity skew in the progeny, we applied contingency tests examining whether multiply mated mother snails used sperm in equal frequencies from different mating partners for egg fertilization.

The frequencies of different types of behaviour of an individual are not independent from each other. We used multivariate analysis of variance (MANOVA) to examine whether snails with different numbers of copulations differed in the percentage of time spent crawling, feeding, digging and resting. We applied principal components analysis (PCA; Legendre & Legendre 1998) to convert the different behaviour types of individuals to uncorrelated variables. The first PCA axis explained well the interindividual variation in the frequency of the different types of behaviour (see Results). In further analyses, we used the coordinates of each individual on the first PCA axis (multiplied by −1) as a measure of activity.

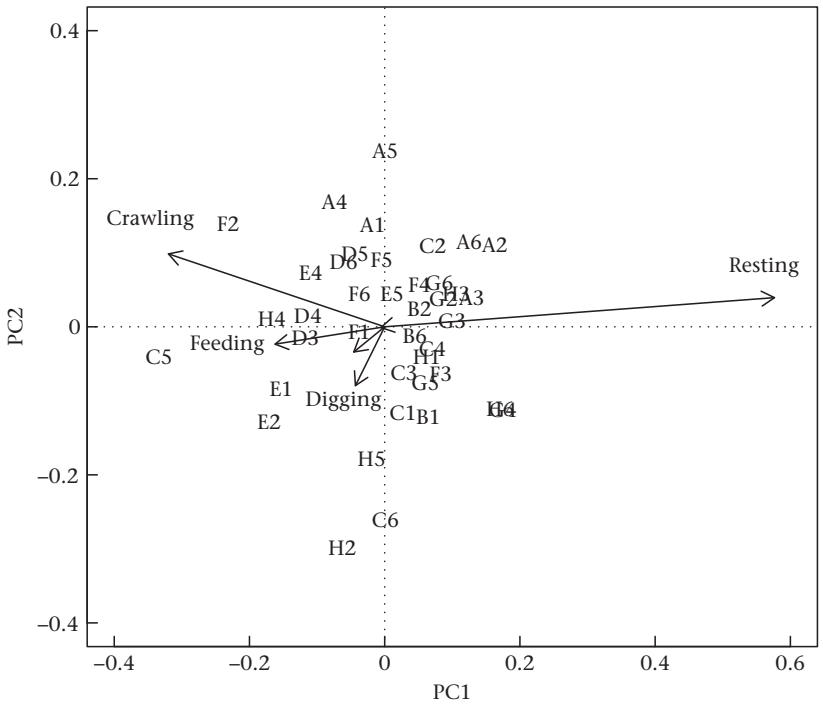

Figure 1. Results of the principal components analysis on the behavioural variability of individual snails ($N = 40$). Types of behaviours are represented by arrows and snails are indicated by the observation chamber (letters A–H) and the individual label (1–6) within chamber. For clarity, only crawling, feeding, digging and resting are displayed.

Correlation analyses showed that activity, distance moved within 24 h and the number of long contacts (square-root transformed) were highly intercorrelated (see Results). We therefore used only activity in further analyses. We applied two-tailed Mantel tests to assess correlations between the number of long contacts and differences in shell size in each copulating pair, between the number of long contacts and genetic distance of two copulating snails, and between the shell size difference and genetic distance of two copulating snails, for each observation chamber separately. Mantel tests were run to calculate Pearson correlation coefficients based on 1000 permutations.

We used generalized linear mixed models (GLMM; Zuur et al. 2009) to examine how activity (A), shell size (S), heterozygosity (square-root transformed, H) and the interaction between activity and shell size ($A*S$) of individual snails influenced their courtship and mating frequency and female and male reproductive success. Observation chambers were handled as a random intercept. We used GLMMs with Poisson distribution because the response variables were zero inflated and are biologically interpretable only as integers. Predictors were centred prior to the analyses. We started with a complete model (main effects plus a two-way interaction). Then the minimal adequate model was selected based on Akaike's information criterion (AIC).

Estimates of female reproductive success were highly accurate because we genotyped 95.5% of the hatchlings and could assign them to their mothers. Estimates of male reproductive success, however, were influenced by two additional factors. First, some snails that had received sperm from our focal snails died during the study. Delivering sperm to these snails reduced the male reproductive success of focal snails. We therefore repeated the GLMM analyses using a reduced data set that considered only those four observation chambers in which no snail died. This revealed a more accurate estimate of male reproductive success for the 24 focal snails considered. Second, snails that copulate successfully may store sperm from their mates to fertilize eggs in the coming reproductive season(s) (Baur 1994b). Thus, long-term sperm storage reduces the accuracy of any estimate of male reproductive success restricted to a single season. The magnitude of this factor cannot be assessed with the design chosen in the present study.

Finally, snails that did not copulate in the study ($N = 19$) could influence the results. To examine this aspect we repeated the GLMM analyses using a reduced data set that considered only snails that copulated ($N = 21$). These analyses revealed very similar results for all main predictors as the overall analyses based on all snails ($N = 40$ for courtship and mating frequency, and $N = 37$ for female and male reproductive success; data not shown).

PCA was performed with the vegan package (Oksanen et al. 2010), GLMM with the lme4 package (Bates & Maechler 2010) and the Mantel test using the ecodist package (Goslee & Urban 2007) in the R statistical environment (R Development Core Team 2009).

RESULTS

Snail Behaviour

Over the entire experimental period of 58 days, the snails on average used 9.9% of their time for crawling, 4.8% for feeding, 0.6% for mating (courtship and copulation), 3.1% for digging (including egg laying) and 81.5% for resting. However, individual snails showed considerable variation in the percentage of time used for different types of behaviour (Fig. 1). PCA was used to convert the nonindependent data on the proportion of different types of behaviour to uncorrelated variables for each individual. The inter-individual variation in frequency of the different types of behaviour could be expressed by the first axis of the PCA, which explains 94.4% of the total variability (Fig. 1). Individuals with negative values on the first PCA axis were highly active (spent much time crawling, feeding and digging), while individuals with positive

values on the first PCA axis were mostly passive (spent much time resting). Further PCA axes explained a low percentage of total variability (values for the second and third axes were 3.9% and 1.3%). We therefore expressed the differences in the behaviour of individuals by the value of the first PCA axis. This value multiplied by −1 can be considered as a measure of activity for each snail (see Statistical Analyses).

Snails with different numbers of copulations differed significantly in the percentage of time spent with various behaviours (Table 1). The number of copulations increased with the amount of time spent crawling and feeding.

Determinants of Mating Success

Individuals varied in number of long contacts with other snails from 21 to 182 in 58 days (mean = 84.8, $N = 40$). The distance moved within 24 h by a snail averaged 3.04 m (range 0.86–6.27 m), resulting in a mean total distance moved of 166.5 m in 58 days (range 47.5–345.8 m, $N = 40$). The number of long contacts a snail had, the distance moved within 24 h and the activity of the individual were highly intercorrelated (Pearson product-moment correlation: number of long contacts [square-root transformed] versus distance moved: $r = 0.74$, $P < 0.001$; number of long contacts versus activity: $r = 0.73$, $P < 0.001$; distance moved versus activity: $r = 0.90$, $P < 0.001$; $df = 38$ in all cases). Mantel tests revealed that the number of long contacts between individuals of copulating pairs were randomly distributed with respect to the difference in shell size (in all seven observation chambers) and with respect to the genetic similarity between mating snails (in six of seven observation chambers; snails from observation chamber B were excluded from these analyses). Furthermore, there was no correlation between the difference in shell size and the genetic similarity in pairs of mating snails (in all eight observation chambers).

On average 2.2% of the long contacts (range 0–13.7%) led to courtship. Individual snails courted between 0 and 14 times (mean = 2.3, $N = 40$). The number of courtships was positively affected by the activity of a snail, but negatively by its shell size (Table 2). The negative interaction between activity and shell size suggests that the effect of activity on the number of courtships is negatively influenced by snail size. On an individual basis, on average 60.3% of the courtships (interindividual range 0–100%) resulted in a copulation.

Five of the 40 snails copulated three times, six copulated twice and 10 once, whereas 19 individuals did not copulate at all during the observation period of 58 days. However, five of the 19 snails with no copulation courted unsuccessfully 1–14 times. Active snails copulated more frequently than passive snails (Table 2). Snails that copulated three times spent 66% more time crawling than snails that copulated once or not at all. Snails that copulated three times spent 107% more time feeding than snails with one or no copulation. Model selection eliminated the factor snail size suggesting that shell size did not influence the number of copulations.

Table 1
Percentage of time allocated to different behaviours in snails with different number of copulations

Type of behaviour	Number of copulations			
	0	1	2	3
Crawling	8.7±3.1	8.0±2.0	13.2±3.6	14.1±4.1
Feeding	4.1±1.1	3.6±1.0	6.1±1.6	8.1±2.1
Courtship	0.25±0.61	0.33±0.18	1.04±0.64	1.82±0.76
Copulation	–	0.15±0.06	0.24±0.09	0.29±0.04
Digging	3.0±1.2	2.8±1.1	3.8±1.2	3.8±0.4
Resting	83.9±4.5	85.2±3.4	75.6±4.5	71.8±6.3
Number of snails	19	10	6	5

Mean ± SD for each measure are shown.
MANOVA: Pillai = 1.399, approximate $F_{3,36} = 4.809$, $P < 0.001$.

Table 2
Summary of the minimal adequate generalized linear mixed models examining the effects of activity, shell size, degree of heterozygosity and the activity*shell size interaction on courtship, copulation and reproductive success as female and male in individual snails

Response variable	Predictor	Estimate	SE	z	P
Number of courtships*	Intercept	0.304	0.284	1.070	0.285
	Activity (A)	7.536	1.129	6.687	<0.001
	Snail size (S)	−0.341	0.133	−2.564	0.010
	$A*S$	−2.743	1.089	2.519	0.012
Number of copulations*	Intercept	−0.346	0.206	−1.678	0.093
	Activity (A)	5.685	1.341	4.240	<0.001
	Heterozygosity†	1.116	0.677	1.650	0.099
Reproductive success as female‡	Intercept	1.092	0.214	5.102	<0.001
	Activity (A)	12.648	0.898	14.086	<0.001
	Snail size (S)	−0.816	0.128	−6.340	<0.001
	Heterozygosity†	3.926	0.399	9.819	<0.001
	$A*S$	2.699	0.727	3.712	<0.001
Reproductive success as male‡	Intercept	1.479	0.223	6.620	<0.001
	Activity (A)	7.415	0.711	10.434	<0.001
	Snail size (S)	−0.305	0.086	−3.536	<0.001
	Heterozygosity†	2.522	0.374	6.742	<0.001

'Estimate' indicates the magnitude and direction (positive or negative) of the effect of predictor variables. Predictors were centred prior to the analyses.
* $N = 40$.
† Square-root transformed.
‡ $N = 37$.

Determinants of Female and Male Reproductive Success

Female reproductive success varied between 0 and 119 hatchlings (median = 0, mean = 13.2, $N = 37$), and was affected positively by both the activity and the degree of genetic heterozygosity of the focal snail and negatively by snail size (Table 2). However, the positive interaction between activity and shell size indicates that the effect of activity was positively influenced by snail size (Table 2). For snails that copulated, female reproductive success averaged 24.4 hatchlings (range 0–119, $N = 20$).

Individual snails sired between 0 and 38 hatchlings (median = 0, mean = 8.7, $N = 37$). The male reproductive success was affected positively by the activity of the individual and its degree of genetic heterozygosity, but negatively by shell size (Table 2). For snails that copulated, male reproductive success averaged 16.1 hatchlings (range 0–38, $N = 20$). Highly skewed paternity patterns were found in the progeny of multiply mated snails. Different fathers sired different numbers of offspring in the progeny of 71.4% of the mother snails that had copulated with two or three different partners (contingency test: $P < 0.05$ in progeny arrays).

The total number of hatchlings emerging from eggs should be equal to the total number of offspring sired in groups of hermaphrodite snails provided that there is no parent mortality during the study and that all offspring can be assigned to their mother and father. In four of eight observation chambers no snail died. The 24 mother snails in these four boxes produced a total of 198 hatchlings of which 195 could be assigned to their fathers (the DNA could not be extracted from three hatchlings). Mean female reproductive success ± SD was 8.2 ± 14.1 hatchlings and male reproductive success averaged 8.1 ± 13.5 hatchlings. GLMM analyses revealed similar results as the analyses based on the entire data set. Female and male reproductive success were affected positively by both the activity (Fig. 2) and the degree of heterozygosity of the focal snail and negatively by its shell size. There was, however, a negative interaction between activity and snail size affecting male reproductive success indicating that the effect of activity was negatively influenced by shell size.

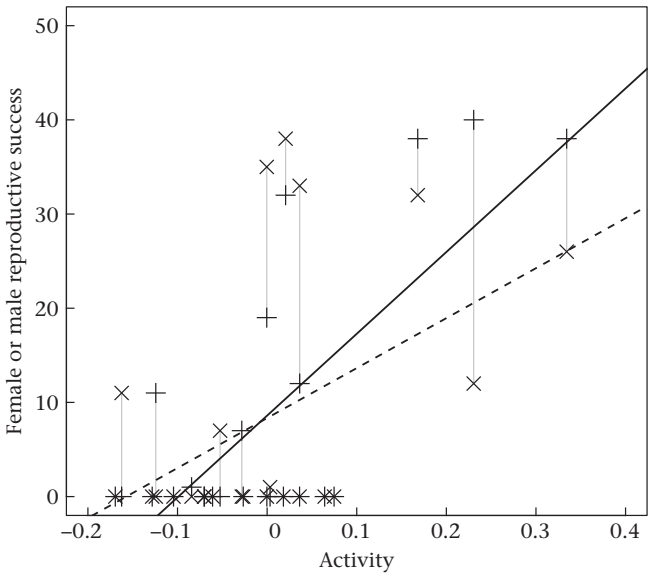

Figure 2. Relationships between activity (A) and both female and male reproductive success (FRS and MRS) of individual snails: +, continuous line: FRS; x, dashed line: MRS. Data from the four observation chambers with no snail mortality are shown. Regression lines are: $FRS = 8.598 + 86.844A$, $R^2 = 0.54$, $N = 24$, $P < 0.001$; $MRS = 8.338 + 53.046A$, $R^2 = 0.22$, $N = 24$, $P = 0.021$. The two regression lines do not differ in slope ($P = 0.22$).

For all snails, female and male reproductive success were positively correlated within the same individual (Pearson correlation, (log+1) − transformed values: $r_{35} = 0.79$, $P < 0.0001$). The positive correlation remained when noncopulating snails were excluded from the analysis ($r_{18} = 0.53$, $P < 0.015$), and when only the 24 individuals of the four observation chambers with no snail mortality were considered ($r_{22} = 0.77$, $P < 0.0001$). It was therefore not surprising that both female and male reproductive success increased with the number of copulations.

DISCUSSION

Our study shows that mating success and both female and male reproductive success in *A. arbustorum* are mainly determined by the activity of the individual snail. In this context, activity is a combined measure of the time spent crawling, feeding and digging. In simultaneous hermaphrodites with reciprocal copulation and mutual sperm transfer, mating success is equal for both gender functions of an individual. This is also true for our model species. Furthermore, we found that female reproductive success was positively correlated with male reproductive success and that both increased with the number of copulations a snail had. To our knowledge this is the first study estimating both female and male reproductive success in a simultaneous hermaphrodite snail kept under seminatural conditions.

Sexual selection models show that simultaneous hermaphroditism will be stable when there is a positive correlation between female and male reproductive success (Charnov et al. 1976; Charnov 1979; Leonard 2006). Our findings support these models. However, our results contradict a basic assumption of sex allocation theory. Based on the simplified assumption that all individuals in a population have the same fixed reproductive resource budget, sex allocation theory predicts a trade-off between female and male reproductive allocation (Charnov 1982). However, there is so far little empirical evidence for this trade-off in animals (Schärer 2009). Studies have often found no correlation (Baeza 2007) or a positive correlation (Schärer et al. 2005) between female and male allocations rather than the expected negative correlation. In many cases, the trade-off assumptions seem not to be justified. Furthermore, the processes that translate the female and male allocations into gender-specific fitness are still poorly understood (Schärer 2009). Locher & Baur (2000a) found that the reproductive allocation was highly female biased in *A. arbustorum* and that an increased mating frequency led to an increased allocation to the male function. However, even snails that copulated three times invested less than 5% of the total energy allocated (expressed as dry weight, nitrogen or carbon content of the released gametes) to the male function. Thus, the findings of Locher & Baur (2000a) and our results indicate that there is no direct link between male allocation and male reproductive success (i.e. a linear male fitness gain curve) in this simultaneous hermaphrodite snail.

Activity as measured in our study might be a proxy for the condition or healthiness of a snail. Individuals of *A. arbustorum* infested by parasitic mites showed reduced activity, produced fewer eggs and exhibited more winter mortality than parasite-free snails (Schüpbach & Baur 2008, 2010). Furthermore, in mating tests individuals with a restricted food supply tended to court for longer and copulated for a shorter period than individuals with ample food supply (Locher & Baur 2002). Thus, the among-individual variation in activity recorded in our study may represent the variation in condition of members of a snail population.

Bateman's principle also applies to hermaphrodites (Charnov 1979; Anthes et al. 2010). Individuals with a more female-biased sex allocation are expected to adopt a mating strategy that is more discriminating and that selects for mating partners that provide the highest direct or indirect benefits (Janicke & Schärer 2009). However, females often mate multiply, most frequently with different mates. Hence, there must be a series of additional benefits to females from mating, besides receiving enough sperm to fertilize all their eggs. Females mating with multiple males may obtain direct benefits such as parental care, nuptial gifts or energy gains from seminal fluid, or indirect (i.e. genetic) benefits resulting in higher-quality offspring (Arnqvist & Rowe 2005). In the simultaneous hermaphrodite *A. arbustorum*, the female function may have multiple benefits from receiving additional sperm such as: (1) reproductive assurance (Chen & Baur 1993); (2) inbreeding avoidance (Chen 1994; Baur & Baur 1997); (3) the opportunity of cryptic female choice when receiving sperm from different mates (Baur 1994b); (4) energy and nutrients gained from resorbing sperm and spermatophore; and/or (5) stimulation of egg production. In *A. arbustorum* as well as in other land snails, repeated mating stimulates egg production, which leads to an increased batch size and/or the production of more batches even later in the reproductive season (Bride et al. 1991; Saleuddin et al. 1991; Baur & Baur 1992). Moreover, the presence of other conspecifics may also affect female fecundity in land snails in different ways. At low population density social facilitation might increase fecundity (Baur & Baur 2000), while at high population density interference competition through mucus trails reduces fecundity (Baur & Baur 1990; Jordaens et al. 2007).

While direct benefits are easily identified, it is difficult to determine indirect benefits, and it is hence largely unclear how they are obtained. This is particularly true in simultaneous hermaphrodites. Genetic benefits raise the fitness of a female's offspring, which is either achieved by chance when females use multiple partners without choosing between them (i.e. female bet hedging to increase the genetic variance of their offspring; Yasui 2001) or by selective use of sperm from high-quality males. Using sperm from different mates for the fertilization of an egg batch increases the genetic diversity among the offspring of that batch. Indeed, two to six fathers were found to contribute to the fertilization of single egg batches laid by *A. arbustorum* in natural populations (Kupfernagel et al. 2010). Given that particular

combinations of maternal and paternal genotypes affect offspring fitness, snails would benefit by making mate choice decisions based on genetic dissimilarity. There is increasing empirical evidence that genetic compatibility is a consequence of female mate preference favouring genetically dissimilar males (Mays & Hill 2004). However, our study design does not allow us to test this hypothesis because snails kept in groups of six individuals had no free choice between all potential partners in the population.

Sex allocation models and other models addressing gender conflicts cannot satisfactorily account for the diversity found in hermaphrodite mating systems (Michiels 1998; Greeff & Michiels 1999; Leonard 2006). Recently, the gender ratio hypothesis has been proposed as a more general framework (Anthes et al. 2006). This hypothesis requires that hermaphrodites assess not only their own need for allosperm as fertility insurance or nutritional input, but also the quality of their current mating partner. In hermaphrodites, advantages in precopulatory mate competition and mate choice decisions have frequently been attributed to body size, with larger individuals being preferred mating partners because body size is often correlated with fecundity (reviewed in Leonard 2006). Size assessment via simple tactile stimuli and size-assortative mating has been found in opisthobranch gastropods, flatworms and earthworms (reviewed in Anthes et al. 2006) but not in pulmonate gastropods (e.g. Baur 1992; Jordaens et al. 2005). In *A. arbustorum*, clutch size and egg size are correlated with shell size (Baur 1988b, 1994a), but not female reproductive output per season (Baur & Raboud 1988) and male fecundity measured by the number of sperm in a spermatophore and sperm length (Baur et al. 1998; Minoretti & Baur 2006). In our study, there was no relationship between the shell sizes of mating partners.

Empirical evidence for partner assessment cues in hermaphrodites is still limited. The extended courtship in pulmonate land snails should provide ample opportunities for partner assessment (Baur 1998). In our study, 2.2% of the long contacts (interindividual range 0–13.7%) led to courtship, and 60.3% of the courtship (interindividual range 0–100%) resulted in copulation, suggesting a multilevel assessment of potential partners in *A. arbustorum*, although the relevant cues are not known. Direct assessment of the risk or intensity of competition with rival sperm is even less well understood. However, controlled mating trials showed that individuals of *A. arbustorum* do not adjust sperm release according to the potential risk of sperm competition incurred with a virgin or a nonvirgin mating partner (Baur et al. 1998). Furthermore, individuals of *A. arbustorum* did not respond to experimentally increased cues from conspecifics, which were designed to mimic a high risk of sperm competition by delivering more sperm (Locher & Baur 2000b).

Analyses of paternity and maternity usually ignore matings that failed to produce offspring (e.g. because of postcopulatory sexual selection or extremely low hatching success). This deficiency can be circumvented by combining parentage analysis with video recording of all potential mothers and fathers as done in our study. We recorded four individuals that mated once and reproduced as male but not as female and another four snails that laid fertilized eggs but did not sire any offspring. As explained in the section Statistical Analyses, assessments of male reproductive success restricted to a single season are truncated because of long-term sperm storage in the spermatheca of mating partners. Thus, male reproductive success is underestimated in our study. Indeed, genetic markers demonstrated that multiply mated individuals of *A. arbustorum* also used sperm received in the preceding year for egg fertilization (Baur 1994b). It is therefore not possible to compare the variance of reproductive success of either sexual function in our study.

Dart shooting could also influence paternity patterns in *A. arbustorum*. In many species of terrestrial gastropods, a sharp, calcified or chitinous dart is used to pierce the body of the mating partner during courtship (Baur 1998). Even though darts may wound a partner, the elaborate structure of the dart apparatus suggests that it serves some adaptive function (Chase 2007). Experimental studies in the land snail *C. aspersum* revealed that the dart transfers a substance that induces conformational changes in the female reproductive tract of the recipient, which reduces sperm digestion and, consequently, increases the chances of storing the dart shooter's sperm (Chase & Blanchard 2006). However, virgin snails do not possess a dart and the frequency of dart shooting varies widely among gastropod species (Chase 2007; Baur 2010). In *A. arbustorum*, dart shooting is optional, occurring in 10–20% of courting, nonvirgin individuals (B. Baur, unpublished data). The video technique used in the present study did not allow us to record dart shooting in all courting pairs (e.g. in those snails mating upside down attached to the glass cover of the observation chamber). Dart shooting might have occurred in a few snails mating for the second or third time, but its low frequency may not change the general findings on male reproductive success.

The number of copulations recorded over 58 days is probably an underestimation of the total number of copulations per year, because individuals of *A. arbustorum* frequently mate after the egg-laying period, sometimes even a few days before hibernation (B. Baur, unpublished data). Sperm received from these matings may be stored until the succeeding season(s) (Kupfernagel et al. 2010). However, we do not believe that late matings in autumn would change our general findings.

In our study, the degree of heterozygosity also explained variation in mating success and in female and male reproductive success. Individuals with a high degree of heterozygosity copulated more frequently and showed greater reproductive success both in the female and male function than snails with a lower degree of heterozygosity. The snails used in our study were collected from a very large (>3000 individuals) natural population. Thus, it seems very unlikely that a high level of inbreeding occurs in this population (Pemberton 2004). Heterozygosity–fitness correlations have been studied in various organisms for a long time, but they are not universal as numerous studies have yielded no correlations (David 1998). However, Britten (1996) concluded from a meta-analysis that heterozygosity–fitness correlations were on the whole significant, and would remain so even when considering a reasonable number of unpublished null results. In a number of populations and species, decreased fertility, survival, and parasite and disease resistance are associated with homozygosity and inbreeding (Young & Clarke 2000). However, the general mechanisms underlying correlations between multilocus heterozygosity and fitness are not well understood (Avise & Hamrick 1995).

To sum up, we have shown that the large interindividual variation in reproductive success can mainly be explained by different degrees of activity of individual snails, and that female and male reproductive success are positively correlated within individuals of this simultaneous hermaphrodite. Our study was restricted to one reproductive season whereas in *A. arbustorum*, individuals reproduce on average over 4 years (maximum 14 years; Baur & Raboud 1988). Hence, studies should assess lifetime reproductive success for both the female and male functions of a snail and check for possible trade-offs between current reproductive success, winter survival and future reproductive success.

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Chapter 2

Variation in multiple paternity and sperm utilization patterns in natural populations of a simultaneous hermaphrodite land snail

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Variation in multiple paternity and sperm utilization patterns in natural populations of a simultaneous hermaphrodite land snail

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Mating frequency has important implications for patterns of sexual selection and sexual conflict, and hence for issues such as the maintenance of genetic diversity and speciation. We assessed the level of multiple paternity and sperm utilization patterns in four natural populations of the simultaneous hermaphrodite land snail *Arianta arbustorum* using four polymorphic microsatellite loci. A total of 1088 offspring from 26 wild-caught snails were genotyped to determine the number of fathers siring each brood and paternity skew in succeeding clutches. Multiple paternity was detected in the offspring of all 26 mother snails examined with the contribution of two to six fathers. The four populations examined differed in the level of multiple paternity. Snails in the population with the highest density of adults showed the highest level of multiple paternity, whereas snails in the population with the lowest density exhibited the lowest value of multiple paternity. Highly skewed paternity patterns were found in the progeny of 15 (57.7%) of the 26 mother snails. The number and identity of fathers siring the offspring of single mothers also varied among successive clutches. Furthermore, genetic analyses indicate a low level of self-fertilization in one of the four populations. *Biological Journal of the Linnean Society*, 2010, **99**, 350–361.

ADDITIONAL KEYWORDS: *Arianta arbustorum* – Gastropoda – mating frequency – paternity analysis – population density – self-fertilization.

INTRODUCTION

The extent of multiple paternity is an important component in the evolution of life history and population genetics. Mating frequency has implications for the effective population size, rate of gene flow, genetic structure of the population, and maintenance of genetic variation (Reed & Frankham, 2003). In males, mate acquisition is frequently the most severe limitation on reproductive success and, consequently, male strategies to ensure mating with multiple females are common and widespread in virtually all animal taxa (Simmons, 2001; Uller & Olsson, 2008). In contrast, female reproductive output in terms of the number of offspring is commonly not limited by the number of mating partners, and selection on multiple mating in females should therefore be weaker than it is in males, especially in species with internal fertilization.

In simultaneous hermaphrodites, selection on female traits cannot be independent of selection on male traits of the same individual. Many simultaneous hermaphrodites mate multiply and store sperm for long periods (Baur, 1998). Consequently, simultaneous hermaphrodites are affected by forces similar to those leading to complicated mating strategies and sperm competition in animals with separate sexes (Charnov, 1996; Michiels, 1998; Anthes, Putz & Michiels, 2006). In helicid snails, mating has been reported to be random with respect to shell size, shell colour, banding pattern, and the degree of relatedness (Lamotte, 1951; Wolda, 1963; Baur, 1992; Baur & Baur, 1997). Individuals of *Helix pomatia*, *Cornu*

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aspersum (formerly *Helix aspersa*), *Cepaea nemoralis*, and *Arianta arbustorum* have been observed to mate repeatedly with different partners in the course of a reproductive season (Wolda, 1963; Baur, 1988; Evanno, Madec & Arnaud, 2005). Using shell colour and banding pattern as genetic markers, paternity analyses in wild-caught individuals of *C. nemoralis* indicated that at least two fathers contributed to the fertilization of the eggs of each clutch (Murray, 1964). Similarly, 63% of a sample of wild-caught individuals of *A. arbustorum* used sperm from at least two males for the fertilization of their eggs (Baur, 1994a). However, the actual frequency of multiple paternity and the number of sires involved in the fertilization of egg clutches might be underestimated by this technique because repeated matings with snails of the same shell colour genotype will produce results that are indistinguishable from the broods of single matings. Accurate data on the number of sires involved in the fertilization of egg clutches in wild land snail populations are so far not available. Research on mating strategies has been hampered by the notorious difficulty with which mating can be reliably observed in wild snail populations. The recent development of molecular techniques and software programs, however, allow paternity assignment in invertebrates with a rather cryptic life.

In the present study, we used microsatellite markers to estimate the frequency of multiple paternity in natural populations of *A. arbustorum*. In the wild, populations of *A. arbustorum* vary widely in density of individuals (< 0.1–42 adults per m^2; Baur, 1986). Snails living in high-density populations may encounter many potential mates (in simultaneous hermaphrodites, each adult individual encountered represents a potential mating partner), whereas those living in less favourable habitats with low population density may encounter few mates. It is expected that egg clutches laid by snails from high-density populations show, on average, a higher degree of multiple paternity than those from low-density populations.

In hermaphrodites, the ability to reproduce by self-fertilization provides some reproductive assurance in cases when the mate encounter rate is extremely low, enhances colonization success, and is an ecogenetic strategy of adaptation to particular patterns of environmental heterogeneity (Levins, 1968). Mixed mating systems, where reproduction occurs by both selfing and outcrossing, are common in plants (Goodwillie, Kalisz & Eckert, 2005), and have also been found in a variety of simultaneously hermaphroditic gastropods (Baur, 1987; Heller, 1993; Jarne & Auld, 2006). It is, however, unclear how frequent obligate selfing or outcrossing occurs in particular species and populations (Heller, 1993; Backeljau, Baur & Baur, 2001). Complete outcrossing is favoured when the effects of inbreeding are pronounced (Lande & Schemske, 1985).

In *A. arbustorum*, cross-fertilization is the dominant mode of reproduction (Chen & Baur, 1993). Breeding experiments showed that 27% of virgin snails prevented from mating produced a few hatchlings by self-fertilization in the second and third year of isolation (Chen, 1994). However, the reproductive success of selfing individuals was less than 2% of that of outcrossing snails. The occurrence of self-fertilization has so far not been demonstrated in any natural population of *A. arbustorum*.

In the present study, individuals of *A. arbustorum* from four natural populations were maintained in the laboratory, where they produced offspring from stored sperm. The mothers and their offspring emerging from successive clutches were genotyped for microsatellite DNA markers at four loci, and the patterns of paternity were reconstructed from their genotypes. Furthermore, we also used the genotypic frequencies at marker loci to estimate selfing rates in the four populations.

MATERIAL AND METHODS

Study organism

Arianta arbustorum is common in moist habitats of north-western and central Europe (Kerney & Cameron, 1979). Individuals become sexually mature at 2–4 years old and adults live for another 3–4 years (Baur & Raboud, 1988). Mating in *A. arbustorum* includes elaborate courtship behaviour, which lasts 2–18 h (Baur & Baur, 1992a); both snails transfer simultaneously one spermatophore. *Arianta arbustorum* mates repeatedly in the course of a reproductive season and fertile sperm can be stored more than 1 year in the sperm storage organ (Baur, 1988). Snails deposit one to three clutches consisting of 20–50 eggs per year (Baur, 1990). The dispersal of marked individuals averaged 7 m in 1 year (Baur, 1986).

Snail samples

Adult individuals of *A. arbustorum* were collected at four sites (hereafter referred to as populations A–D) in the subalpine forest of Gurnigel, Switzerland (46°45.5′N, 7°27.5′E; elevation 1230–1280 m a.s.l.) early in June, approximately 4 weeks after hibernation (Fig. 1). In the Gurnigel forest, *A. arbustorum* is abundant in small clearings with wet soil, along brooks, and in the embankments of tracks constituting a kind of metapopulation (Akçakaya & Baur, 1996). The populations chosen occupied habitats with similar vegetation and climatic conditions. The distance between sites ranged from 100 m (populations B and C) to 1200 m. *Arianta arbustorum* begins to

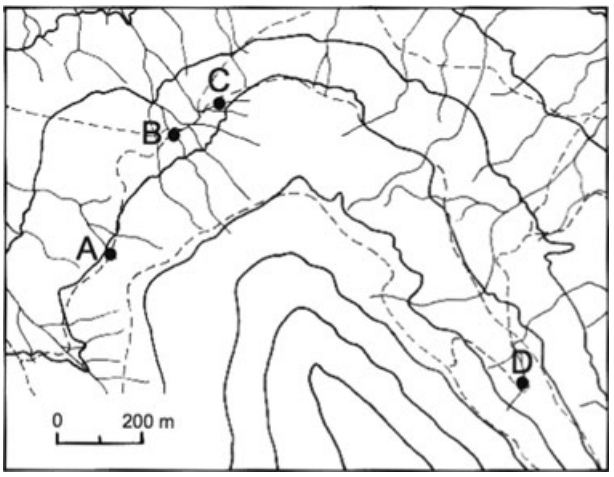

Figure 1. Locations of the four populations of *Arianta arbustorum* examined in the subalpine forest Gurnigel, Switzerland. Snails occur along streams and in the embankments of forest roads and tracks (dashed lines). Intervals of contour lines are 50 m.

mate after arousal from hibernation (Baur, 1992). Thus, the snails used in the present study might have stored allosperm from matings in the previous and ongoing year. The date of snail sampling coincided with the beginning of the egg laying period in the field. The paternity patterns recorded with this experimental procedure may therefore represent the natural situation during the peak period of oviposition.

The snails were kept isolated in transparent beakers (depth 8 cm, diameter 6.5 cm) lined with moist soil (approximately 4 cm) at 19 °C under a 16 : 8 h light/dark cycle. Fresh lettuce was provided twice a week and at the same time the beakers were cleaned. The beakers were checked for eggs once per week. The eggs of each clutch were collected, counted, and kept in a plastic dish (diameter 6.5 cm) lined with moist paper towelling at 19 °C to determine hatching success. Newly-hatched snails were separated from remaining unhatched eggs to prevent egg cannibalism (Baur, 1994b). Hatchlings labelled in order of emergence were stored at –80 °C. Eggs were collected over a period of 60 days. The length of this period corresponds to approximately one reproductive season of *A. arbustorum* living in subalpine forests (Baur, 1990).

Paternity analysis

DNA of individual hatchlings was extracted following a DNeasy protocol of Qiagen (2006). DNA of mother snails was isolated from 20–30 mg foot tissue using a modified CTAB method (Winnepenninckx, Backeljau & De Wachter, 1993). We screened the DNA of mother snails for microsatellite repeats using the nine primers pairs developed by Armbruster, Koller & Baur (2005). Primers that did not amplify or produced only a small number of alleles were no longer considered. Four highly variably microsatellite loci (24, 26, 55, and A9, Table 1) were employed for paternity analyses. Polymerase chain reaction (PCR) mixtures (HotStarTaq Mastermix Kit, Qiagen AG) comprised 4–6 ng of DNA (3 μL) in a total volume of 10 μL in accordance with the protocol of Qiagen (2005). PCR-mixtures were preheated at 95 °C for 15 min, followed by 30–35 cycles of 95 °C for 30 s, locus specific annealing for 30 s, and 72 °C for 30 s (Armbruster, Koller & Baur, 2005). PCR was finished with an extension of 8 min at 72 °C. Horizontal electrophoresis was performed with SEA2000™ advanced submerged gel electrophoresis equipment. Spreadex EL400-gels (Elchrom Scientific AG) were used.

Table 1. Summary statistics for four microsatellite loci used on *Arianta arbustorum* populations

Locus	N	H_O	H_E	Exclusion probability*
24	7	0.69	0.61	0.47
26	5	0.89	0.65	0.42
55	8	0.69	0.70	0.50
A9	7	0.88	0.75	0.50

Data are based on 26 mother snails.
Number of alleles per locus (N), observed heterozygosity (H_O), expected heterozygosity (H_E).
*Combined exclusion probability for all four loci = 0.94.

Null alleles can significantly affect estimates of genetic relatedness of individuals and population genetic structure (Pemberton *et al.*, 1995; Dakin & Avise, 2004). We genotyped 1183 hatchlings from 26 mother snails (population A: $N = 6$ mothers; B: $N = 7$; C: $N = 6$; D: $N = 7$), which produced 78 clutches (Table 2). Null alleles were detected in 95 (8.0%) of the 1183 hatchlings examined. In 44 hatchlings, null alleles were a result of low DNA concentration and, in 51 hatchlings, a result of PCR failure. The frequency of null alleles as a result of PCR failure did not differ among the four primers used ($\chi^2 = 5.43$, d.f. = 3, $P = 0.14$). Data from the 95 hatchlings were omitted. Thus, in the paternity analysis, a total of 1088 hatchlings were considered. This corresponds to 84.7% of all hatchlings produced by the 26 mother snails.

Population density

We estimated the population size of adult *A. arbustorum* in the four sites using a mark–recapture pro-

Table 2. Number of fathers contributing to the offspring of mothers of *Arianta arbustorum* collected in four natural populations (A–D), and variation in multiple paternity in successive clutches

Population, mother	Number of hatchlings analysed	Minimum number of fathers*	Total number of fathers†	Number of hatchlings analysed in subsequent clutches (number of fathers)
A2	49	3	5	22 (5); 12 (3); 15 (4)
A3	27	2	3	14 (2); 13 (3)
A8	27	3	3	5 (2); 11 (3); 11 (2)
A10	36	3	3	4 (2); 3 (2); 15 (3); 14 (3)
A14	58	2	2	12 (2); 14 (2); 23 (2); 9 (2)
A15	58	4	7	21 (7); 22 (7); 15 (5)
Population mean	*42.5*	*2.8*	*3.8*	
B1	101	> 5	12	37 (12); 22 (11); 21 (9); 20 (10); 1 (1)
B6	38	4	6	14 (6); 12 (5); 1 (1); 11 (5)
B9	37	3	5	22 (5); 15 (3)
B10	20	4	6	6 (5); 14 (6)
B12	17	3	4	4 (2); 13 (4)
B13	41	> 6	7	13 (6); 7 (5); 21 (6)
B14	47	4	5	2 (1); 24 (5); 14 (3); 7 (4)
Population mean	*43.0*	*4.1*	*6.4*	
C3	55	5	5	13 (3); 15 (4); 16 (4); 11 (4)
C6	41	> 5	7	11 (4); 30 (7)
C7	53	> 5	6	7 (4); 15 (5); 16 (5); 15 (5)
C9	39	3	5	11 (4); 17 (5); 11 (5)
C14	53	> 6	8	22 (5); 31 (6)
C15	69	5	9	29 (8); 19 (7); 11 (3); 10 (3)
Population mean	*51.7*	*4.8*	*6.7*	
D1	27	3	4	7 (3); 2 (2); 9 (3); 9 (3)
D3	26	3	4	12 (4); 14 (4)
D8	36	3	4	20 (4); 13 (4); 3 (3)
D11	16	2	3	16 (3)
D12	19	2	3	19 (3)
D13	45	5	9	14 (9); 10 (5); 11 (5); 10 (4)
D15	53	3	7	12 (7); 30 (7); 11 (5)
Population mean	*31.7*	*2.9*	*4.9*	

*Parentage reconstruction using GERUD.
†Likelihood assessment based on genetic diversity using COLONY.

cedure. Searches for snails were conducted under conditions favourable for snail activity (moist vegetation and between 07.00 and 10.00 h). All adult snails found were counted, marked on their shells with two small dots of car lacquer and immediately released at the same spot. Two weeks after marking, the sites were searched again for snails with the same searching effort. In the period between release and recapture, the weather was favourable for snail activity, allowing a mixing of marked and unmarked animals. Population sizes at the four sites were estimated with the method of Petersen (Begon, 1979). The numbers of snails collected for multiple paternity assessment prior to the mark–recapture procedure were added to the estimated population sizes. The area inhabited by *A. arbustorum* in the four populations A–D was measured to the nearest m^2 (A: 30 m^2; B: 24 m^2; C: 32 m^2; D: 50 m^2). Local population density is expressed as number of adult *A. arbustorum* per m^2.

Statistical analysis

Microsatellite characteristics were assessed using both GenAlEx, version 6.1 (Peakall & Smouse, 2006), a Microsoft Excel add-on developed for population genetic data analyses, and GENEPOP (Raymond & Rousset, 1995; http://genepop.curtin.edu.au). Statistical power of paternity analysis was assessed by calculating the probability of detecting multiple paternity using PrDM (Neff & Pitcher, 2002).

Sibship analysis and parentage reconstruction of the field-collected mother snails and their progeny were performed with GERUD, version 2.0 (Jones, 2005) and COLONY, version 2.0 (Wang, 2004). GERUD uses multiple-locus data for reconstruction of the contributing paternal genotype(s) from mother–progeny arrays. The software does not distinguish between potential fathers of the same genotype. Consequently, the number of paternal genotypes estimated is equal to a minimum number of involved fathers. The maximum likelihood software COLONY (Wang, 2004) was used to assess the total number of fathers contributing their gametes to a progeny array. For the paternity assignment, the maternal genotypes of the metapopulation were used. The maternal genotypes represent also the potential paternal genotypes in this simultaneous hermaphrodite. COLONY (Wang, 2004) provides the most probable configuration of paternity including assignments of every offspring to one of the estimated paternal genotypes. We calculated individual based rarefaction curves using EstimateS, version 8.0 (Colwell, 2005) to examine whether the populations differed in level of multiple paternity when the same number of offspring were analysed.

Sperm utilization patterns in successive clutches produced by single mothers were analysed in two ways. First, we examined whether multiple fathers divide fertilization equally in a given mother using contingency tests. Second, we determined whether the reproductive success of each father remains constant across clutches. We used simulation software to calculate the expected fertilization probability of each father using all hatchlings analysed of a mother [only clutches with more than ten analysed hatchlings ($N = 46$ clutches) were considered; Table 2], and tested whether the actual probability of paternity differed from the expected one in each clutch (1000 runs). $P < 0.05$ indicates that the proportion of offspring fertilized by a single father differs across clutches.

Inbreeding and the rate of self-fertilization were quantified using both a population structure approach (PSA) and a progeny array approach (PAA). The population structure approach relies on single-generation samples and produces estimates that integrate the inbreeding history over several generations. PSA was performed with RMES (David *et al.*, 2007), which estimates rates of self-fertilization from the distribution of multilocus heterozygosity in population samples. RMES is less sensitive to technical bias such as null alleles than classical single locus inbreeding coefficients or previous multilocus approaches (Jarne & David, 2008). The PAA is based on the comparison between offspring and mother genotypes. PAA presents only recent mating events of the mothers studied. PAA was calculated using the maximum likelihood model implemented in MTLR, version 3.2 (Ritland, 2002). This approach allows estimates of other mating system parameters such as the occurrence of inbreeding (based on the comparison of multilocus population outcrossing rates to single locus outcrossing rates, $t_m - t_s$). The two approaches provide complementary information.

All statistical analyses were conducted using SPSS, version 13.0 (SPSS Inc), unless otherwise noted.

RESULTS

Microsatellite variation

We considered the 26 mother snails from the four populations as the reference sample of the metapopulation. The four loci used in this study varied in the level of polymorphism from five to eight alleles (Table 1). Genotype frequencies at all loci were within expectations of Hardy–Weinberg equilibrium ($P > 0.05$), and no evidence of genotypic disequilibrium between pairs of loci was found ($P > 0.05$). Paternity exclusion probability for all four loci combined was 0.74–0.94 (GenAlEx estimates). Probabilities of detecting multiple paternity were $\geq 94\%$ for all clutches when assuming equal paternal contributions of the involved fathers, and $\geq 91\%$ when assuming skewed paternal contribution.

Variation in multiple paternity

Microsatellite data of all four loci were considered for 1088 offspring from 26 mother snails (Table 2). The proportions of offspring included in the paternity analyses averaged 84.7% (range: 60.0% –98.1%) of the total number of hatchlings produced (Table 2).

Multiple paternity was found in the offspring of all mother snails (Table 2). The level of multiple paternity, however, was highly variable among snails. The most conservative paternity estimate, the minimum number of fathers, obtained from all loci with GERUD, version 2.0, was in the range 2–6 (mean ± SD: 3.7 ± 1.2) (Table 2). The estimates of most likely numbers of fathers from COLONY, version 2.0, ranged from 2–12 contributing males per mother snail (mean ± SD: 5.5 ± 2.3) (Table 2). The estimates of contributing fathers by the two methods matched relatively well (Spearman rank correlation, $r_s = 0.81$, $N = 26$, $P < 0.0001$), considering that the first method estimates only the minimum number of fathers, whereas the most probable number of sires given by the second method should be higher than the minimum (Table 2). The number of fathers per offspring array was positively correlated with the number of offspring analysed ($r_s = 0.53$, $N = 26$, $P = 0.005$). However, the number of fathers per off-

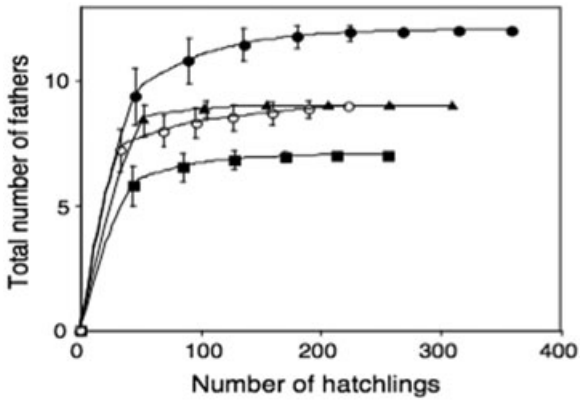

Figure 2. Rarefaction curves for the number of fathers (COLONY estimates) siring hatchlings of single mothers of *Arianta arbustorum* in the four populations: A (squares), B (full dots), C (triangles), and D (open dots). The SD is indicated.

spring array was not correlated with the proportion of hatchlings analysed per mother–offspring array ($r_s = 0.30$, $N = 26$, $P = 0.14$).

Independent of the method of estimate, the four populations examined differed in the level of multiple paternity (GERUD: $F_{3, 22} = 5.04$, $P = 0.008$; COLONY: $F_{3, 22} = 3.59$, $P = 0.03$) (Table 2). Rarefaction curves showed that the four populations also differed in the level of multiple paternity when the same number of offspring were analysed (Fig. 2).

The populations differed in the density of adults: population A 7.7 ± 1.2 (SE) snails per m^2; population B 7.6 ± 1.1 snails per m^2; population C 10.9 ± 1.6 snails per m^2; and population D 1.7 ± 0.3 snails per m^2. Snails in population C with the highest density of adults showed also the highest level of multiple paternity, whereas snails in population D with the lowest density exhibited the lowest level of multiple paternity (GERUD estimate).

Patterns of sperm utilization

Different fathers sired different numbers of offspring in the progeny of 15 (57.7%) of the 26 mother snails (Appendix 1, contingency-test, in 15 cases, $P < 0.05$). In the progeny of the remaining 11 mother snails the different fathers sired equal numbers of offspring ($P > 0.05$). In population A, highly skewed paternity patterns were found in the progeny of all mother snails. Furthermore, single fathers sired more than 50% of the offspring of four of the six mother snails in population A (Appendix 1). In the other populations, no single father sired 50% or more of the offspring of a mother snail. The number and identity of fathers siring the offspring of single mothers also varied among successive clutches (Table 2, Appendix 1). In 38 clutches, the proportions of contributing fathers corresponded to the overall proportions of the different fathers siring the offspring of a single mother snail (simulation test, in all cases, $P > 0.05$). In a further eight clutches (17.4%), however, the paternity analysis revealed a significant deviation from random sperm utilization with respect to all the hatchlings of a mother snail (simulation test, in eight clutches $P < 0.05$), suggesting selective sperm use.

Rate of self-fertilization

Estimations of selfing rates and biparental inbreeding (i.e. mating between close relatives) are given in Table 3. The PSA, which reflects the long-term mating history, revealed evidence for a low (not significant) overall self-fertilization rate, when the mother snails of all populations were considered ($P = 0.07$). At the population level, selfing was found to occur in population A ($P = 0.03$), whereas, in the remaining populations, no evidence for self-fertilization was found. The PAA, which reflects only recent mating events of the maternal individuals, revealed no evidence for self-fertilization, with one exception being the slight tendency for selfing found in population A. The overall level of inbreeding was relatively low (Table 3). However, moderate levels of inbreeding were found in population A (which is consistent with a low rate of self-fertilization) and in population D (the site with the lowest population density) (Table 3).

DISCUSSION

Multiple paternity

The present study provides evidence for a high level of multiple paternity in wild populations of *A. arbustorum*. Furthermore, the four populations examined differed with respect to the level of multiple paternity. Repeated copulations have been reported in several helicid snail species. *Helix pomatia* copulated two to six times per year in a Danish population (Lind, 1988) and two to four times in a German population (Tischler, 1973), and *C. aspersum*, on average, copulated three times (a maximum of seven times) in a British population (Fearnley, 1993, 1996). This results in multiple paternity if a snail copulates with different partners (Evanno *et al.*, 2005). However, copulation does not necessarily indicate successful sperm transfer to the storage organ of the receiver. Some snails may fail to deliver a spermatophore and, in other cases, the receiver may not store any sperm from the mating partner (Chen & Baur, 1993; Haase & Baur, 1995). After successful copulation in *H. pomatia* and *C. aspersum*, only a tiny fraction of the allosperm received is stored in the spermatheca (0.02–0.20%)

Table 3. Quantified self-fertilization and biparental inbreeding rates in natural populations of *Arianta arbustorum*, assessed by the long-term mating history (population structure approach; PSA) and recent mating events of mothers (progeny array approach; PAA).

	PSA‡			PAA†					
Population	N_m	S (g_2)	P	N_h	S (F)	F	t_m	t_s	$t_m - t_s$
A	6	0.28	0.03	255	0.17	0.08	0.65*	0.43*	0.22*
B	7	0.00	0.15	301	–0.33	–0.20	0.97	0.89*	0.07
C	6	0.03	0.33	310	–0.33	–0.20	0.96	0.81*	0.15*
D	7	0.00	0.58	222	–0.33	–0.20	0.89*	0.63*	0.26*
Total	26	0.09	0.07	1088	–0.26	–0.15	0.86*	0.67*	0.19*

†Calculated with MTLR (Ritland, 2002; 1000 bootstraps).
‡Calculated with RMES (David *et al.*, 2007; 10 000 iterations).
N_m, number of mothers genotyped; S, selfing rate; P, probability to reject the null hypothesis: $g_2 = S = 0$; g_2, second-order heterozygosity disequilibrium; N_h, number of hatchlings genotyped; F, single locus inbreeding coefficient of maternal parents; t_m, multilocus population outcrossing rate; t_s, single locus population outcrossing rate; $t_m - t_s$, extent of inbreeding in the populations examined; asterisks indicate estimates significantly larger than 0 ($t_m - t_s$) or smaller than 1 (t_m and t_s).

(Lind, 1973; Rogers & Chase, 2001). The vast majority of allosperm is transferred into the sperm-digesting bursa copulatrix.

Evidence for multiple paternity in helicid snails has been obtained from controlled mating experiments (*C. aspersum*: Rogers & Chase, 2002; Evanno *et al.*, 2005) and in wild populations using shell colour as genetic marker (Murray, 1964; Baur, 1994a). The present study is, however, the first to quantitatively assess the number of fathers involved in the progeny of single mothers of terrestrial snail species. Our paternity estimates in *A. arbustorum* (3.7 fathers by GERUD and 5.5 by COLONY) were similar to that reported in the marine prosobranch snail *Littorina obtusata* (four to six males; Paterson, Partridge & Buckland-Nicks, 2001) and slightly lower than those in *Littorina saxatilis* (three to eight males by GERUD and four to 10 males by COLONY; Mäkinen, Panova & André, 2007).

The adaptive significance of multiple paternity by females can be explained by several hypotheses: sperm replenishment, nutritional benefits from repeated matings by receiving nutrients with the spermatophore, and genetic advantages (multiple mating with different partners may lead to multiple parentage and thus increased genetic variability within a brood). The results of previous laboratory experiments showed that, in *A. arbustorum*, one copulation per year is sufficient to fertilize all the eggs produced in one reproductive season (Chen & Baur, 1993). However, egg production in *A. arbustorum* tends to increase with the number of matings (Baur & Baur, 1992b). As in other stylommatophoran and basommatophoran snails, mating behaviour and/or the transfer of the spermatophore stimulates egg production via hormones in *A. arbustorum* (Saleuddin, Griffond & Ashton, 1991; Baur & Baur, 1992b; Koene & Ter Maat, 2004). Possibly, prostate fluids affect egg production of the sperm receiver by stimulating the albumen gland to produce albumen or by stimulating egg maturation.

We found that the level of multiple paternity increased with the number of offspring produced by a mother. Previous studies revealed a positive relationship between the number of sperm delivered and the number of eggs produced by individuals of *A. arbustorum* (Locher & Baur, 2000, 2002). Furthermore, in a laboratory experiment, the most active snails copulated more frequently and deposited more eggs than less active individuals (N. Minoretti, unpubl. data). Thus, the most active and most productive snails encounter many mating partners and may receive sperm from different mates.

The local density of potential mating partners is expected to influence the level of multiple paternity. We found the highest level of multiple paternity (GERUD estimate) in the population with the highest density of adults and the lowest level of multiple paternity in the population with the lowest adult density. The population examined occurred in similar habitats situated at the same altitude. However, we cannot exclude that slight among-site differences in environmental conditions influence snail activity to a different extent resulting in different levels of multiple paternity in *A. arbustorum*.

Patterns of sperm utilization

In the present study, skewed paternity patterns were found in the progeny of more than half of the mother

snails. The morphology of the sperm storage organ (spermatheca) may influence the outcome of sperm competition. *Arianta arbustorum* shows a considerable variation in the structure of the spermatheca (Haase & Baur, 1995). It consists of two to nine blind tubules uniting to a common duct, which opens into the fertilization chambers (Baminger, Locher & Baur, 2000). Incoming sperm are not equally distributed among all tubules of the sperm storage organ, suggesting that sperm from different partners might be stored and, in a further step, also might be used separately (Haase & Baur, 1995; Bojat & Haase, 2002). Indeed, there is evidence that the sperm recipient might influence paternity after multiple mating (Baur, 1994a, 1998).

The complex muscular network of the tubules and its innervation indicate that the spermathecal muscles are capable of finely tuned movements with agonistic and antagonistic interactions (Bojat, Dürrenberger & Haase, 2001a). The muscular arrangement indicates that the action of muscles is more important for the extrusion of sperm from the spermatheca than for the uptake and distribution of sperm among the tubules. Bojat *et al.* (2001a) suggested that the distribution of allosperm among the tubules can only be accomplished by the ciliation of the spermathecal common duct. During sperm transfer, these long cilia also prevent autosperm from entering the spermatheca, where they (similar to allosperm) would be capacitated (Bojat, Sauder & Haase, 2001b), resulting in self-fertilization, which can occur in exceptional situations (Chen, 1994).

Precopulatory processes could also influence patterns of allosperm storage. During courtship, many helicid snails attempt to pierce the body walls of their mating partners with mucus-coated calcareous darts (Koene & Schulenburg, 2005). The mucus covering of the dart induces conformational changes in the female reproductive tract of the recipient, closing off the entrance to the gametolytic bursa copulatrix. In *C. aspersum*, a species with obligatory dart shooting, individuals that were hit by darts stored significantly more sperm than did snails that were missed (Rogers & Chase, 2001; Chase, 2007). However, dart shooting is not an obligatory courtship element in *A. arbustorum*. In laboratory matings, 50% of the copulating individuals pushed or tried to push a dart into their partner (Bojat & Haase, 2002). This indicates that the importance of dart shooting for allosperm storage varies among snail species. The skewed paternity patterns recorded in the present study might be a result of precopulatory processes, competition among sperm from different partners, and/or selective storage and use of allosperm from the receiver.

Self-fertilization

The gonads of pulmonate gastropods produce simultaneously both sperm and eggs. Mature sperm are pressed on to the hermaphrodite duct, whereas mature eggs remain in the gonad. For fertilization, eggs have to pass through the hermaphrodite duct with its stored, incapacitated autosperm to reach the fertilization chamber, where they encounter the capacitated allosperm released from the spermatheca (Tompa, 1984). Therefore, incapacitation of sperm in the hermaphrodite duct might be important for preventing self-fertilization (Bojat *et al.*, 2001b). However, under certain circumstances self-fertilization may occur. Lang (1911) reported on occurrence of selfing in extensive breeding experiments with *Cepaea hortensis* and *C. nemoralis*. Some individuals of *A. arbustorum* kept isolated for 2 and 3 years also produced a few self-fertilized eggs (Chen, 1994).

The capacity to self-fertilize is obviously advantageous: offspring can be produced even when mates are unavailable. Reproductive assurance has therefore been a popular hypothesis for explaining the evolution of selfing rate (Jarne & Charlesworth, 1993; Henry *et al.*, 2005). There is, however, a trade-off between the automatic advantage (50%) of gene transmission under selfing and the lowered fitness of the selfed compared to outcrossed progeny (i.e. inbreeding depression). It has been assumed that reproduction in *A. arbustorum*, as well as in *C. nemoralis* and *C. aspersum*, occurs primarily through outcrossing, probably because of strong inbreeding depression (Chen, 1993). The results obtained in the present study confirm this assumption, with a population-level average outcrossing rate of 0.86. Interestingly, the present study also provides evidence for the first time regarding a low rate of self-fertilization in a natural population of a helicid land snail. However, we found no association between the rate of self-fertilization and local population density. It must be emphasized that our estimate of the probability of mate encounter was based on a short-term density estimate. This did not capture the occurrence of much rarer events of low mate encounter probability, that is, as a result of extreme disturbance events (e.g. erosion of stream embankment after heavy rains; Baur, 1986) or very rare founding events. Those events might be the ecological factors maintaining the capacity to self-fertilize as a reproductive assurance mechanism. The mating system of *A. arbustroum* would rapidly revert to preferential outcrossing when mates become available because of avoiding inbreeding depression.

The selfing rate in hermaphroditic gastropods varies among families, populations, and species (Backeljau *et al.*, 2001; Trouvé *et al.*, 2003; Henry *et al.*, 2005). This variation might be influenced by

different factors, including the probability of mate encounter, the magnitude of inbreeding depression, historical events, the reproductive biology, and life history of the species, the prevailing environmental conditions and seasonal aspects (Jarne & Auld, 2006). The particular impact of single factors on the self-fertilization rate in *A. arbustorum* and other helicid snails remains to be disentangled.

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APPENDIX 1

Patterns of paternity in offspring of 26 mothers of *Arianta arbustorum* from four populations. The number of hatchlings sired by different fathers (indicated by different letters) are presented for subsequent egg clutches (data in parentheses) and for all offspring of each mother snail. The same letter in different mother–offspring arrays does not refer to the same father.

Population, mother	Number of hatchlings genotyped	Total number of fathers*	Number of hatchlings sired by different fathers in subsequent clutches*	Total number of hatchlings sired by different fathers*
A2	49	5	(6a, 3b, 9c, 1d, 3e); (6a, 4c, 2d); (7a, 1b, 3c, 4d)	19a, 4b, 16c, 7d, 3e
A3	27	3	(5a, 9b); (4a, 7b, 2c)	9a, 16b, 2c
A8	27	3	(2a, 3b); (9a, 1b, 1c); (8a, 3c)	19a, 4b, 4c
A10	36	3	(2a, 2b); (1a, 2b); (1a, 9b, 5c); (1a, 11b, 2c)	5a, 24b, 7c
A14	58	2	(7a, 5b); (11a, 3b); (16a, 7b); (8a, 1b)	42a, 16b
A15	58	7	(3a, 3b, 6c, 4d, 1e, 2f, 2g); (2a, 7b, 5c, 3d, 2e, 1f, 2g); (3b, 4c, 3d, 4e, 1g)	5a, 13b, 15c, 10d, 7e, 3f, 5g
Population mean	*42.5*	*3.8*		
B1	101	12	(5a, 6b, 3c, 6d, 3e, 3f, 2g, 1h, 2i, 3j, 1k, 2l); (2a, 4b, 1c, 4d, 2e, 2f, 2g, 1h, 1i, 1k, 2l); (4a, 3b, 5d, 1e, 3f, 1g, 2j, 1k, 1l); (3a, 2b, 1c, 1d, 1e, 3g, 3h, 1i, 3j, 2l); (1j)	14a, 15b, 5c, 16d, 7e, 8f, 8g, 5h, 4i, 9j, 3k, 7l
B6	38	6	(5a, 1b, 4c, 1d, 1e, 2f); (3a, 1b, 2d, 3e, 3f); (1c); (5b, 1c, 3d, 1e, 1f)	8a, 7b, 6c, 6d, 5e, 6f
B9	37	5	(6a, 5b, 7c, 3d, 1e); (5a, 5b, 5c)	11a, 10b, 12c, 3d, 1e
B10	20	6	(2a, 1b, 1c, 1d, 1e); (1a, 2b, 4c, 2d, 2e, 3f)	3a, 3b, 5c, 3d, 3e, 3f
B12	17	4	(3a, 1b); (1a, 5b, 5c, 2d)	4a, 6b, 5c, 2d
B13	41	7	(2a, 2b, 2c, 3d, 2e, 2f); (2b, 1c, 2d, 1f, 1g); (3b, 2c, 7d, 1e, 6f, 2g)	2a, 7b, 5c, 12d, 3e, 9f, 3g
B14	47	5	(2a); (4a, 4b, 5c, 6d, 5e); (4a, 1c, 9d); (1b, 3c, 2d, 1e)	10a, 5b, 9c, 17d, 6e
Population mean	*43.0*	*6.4*		
C3	55	5	(5a, 3b, 5c); (1a, 5b, 2c, 7d); (5b, 3c, 6d, 2e); (2b, 2c, 5d, 2e)	6a, 15b, 12c, 18d, 4e
C6	41	7	(3a, 4b, 3c, 1d); (3a, 4b, 7c, 2d, 6e, 6f, 2g)	6a, 8b, 10c, 3d, 6e, 6f, 2g
C7	53	6	(2a, 2b, 1c, 2d); (2a, 1b, 5c, 4e, 3f); (2a, 5b, 4c, 3e, 2f); (2a, 2b, 2c, 6e, 3f)	8a, 10b, 12c, 2d, 13e, 8f
C9	39	5	(5a, 3b, 1c, 2d); (2a, 8b, 2c, 3d, 2e); (1a, 3b, 3c, 1d, 3e)	8a, 14b, 6c, 6d, 5e
C14	53	8	(5a, 1b, 7c, 3d, 6e); (7b, 8d, 1e, 7f, 4g, 4h)	5a, 8b, 7c, 11d, 7e, 7f, 4g, 4h
C15	69	9	(7a, 5b, 1c, 3d, 7e, 3f, 2g, 1h); (1a, 2b, 2d, 1e, 6f, 2g, 5i); (4d, 4g, 3i); (1d, 6g, 3i)	8a, 7b, 1c, 10d, 8e, 9f, 14g, 1h, 11i
Population mean	*51.7*	*6.7*		
D1	27	4	(2a, 2b, 3c); (1b, 1c); (2a, 2b, 5c); (5b, 2c, 2d)	4a, 10b, 11c, 2d
D3	26	4	(5a, 4b, 2c, 1d); (1a, 6b, 5c, 2d)	6a, 10b, 7c, 3d
D8	36	4	(11a, 6b, 2c, 1d); (5a, 3b, 3c, 2d); (1a, 1c, 1d)	17a, 9b, 6c, 4d
D11	16	3	(7a, 6b, 3c)	7a, 6b, 3c
D12	19	3	(9a, 5b, 5c)	9a, 5b, 5c
D13	45	9	(2a, 3b, 2c, 2d, 1e, 1f, 1g, 1h, 1i); (2a, 2b, 3c, 1d, 2i); (4a, 3b, 2f, 1g, 1i); (2b, 2c, 4f, 2g)	8a, 10b, 7c, 3d, 1e, 7f, 4g, 1h, 4i
D15	53	7	(2a, 2b, 1c, 1d, 2e, 3f, 1g); (1a, 1b, 4c, 5d, 9e, 5f, 5g); (1b, 2d, 2e, 4f, 2g)	3a, 4b, 5c, 8d, 13e, 12f, 8g
Population mean	*31.7*	*4.9*		

*Likelihood assessment based on genetic diversity using COLONY.

Chapter 3

Sperm utilization in subadult and adult simultaneous hermaphrodite snails mating in the wild

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Sperm utilization in subadult and adult simultaneous hermaphrodite snails mating in the wild

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Abstract: In species with multiple mating and long-term sperm storage, males are expected to show a preference for mating with virgin and young females to reduce the risk of sperm competition. In various simultaneous hermaphrodite land snail species, sperm production precedes egg production by 2–4 weeks, resulting in a short period of protandric hermaphroditism before shell growth is completed. In a natural population, we collected copulating pairs of the simultaneous hermaphrodite land snail *Arianta arbustorum* (L., 1758) consisting either of two adults, of two subadults, or of one adult and one subadult snail, and determined the paternity of their hatchlings that emerged from subsequently deposited eggs. Adult snails used sperm received from subadult mating partners for egg fertilization in the same frequency as sperm from adults, indicating that subadult and adult snails do not differ in male function. Furthermore, an unfinished shell is not a reliable indicator for virginity, because 35% of the subadult individuals had already sperm stored from previous mating(s). Compared with adults, young individuals exhibited a lower risk of sperm competition, indicated by a higher last mate sperm precedence. However, subadult snails produced fewer eggs than adult snails, counteracting the evolutionary advantage of preferring a young partner with low sperm competition risk.

Résumé : Chez les espèces à accouplements multiples et à stockage à long terme des spermatozoïdes, on s'attend à ce que les mâles montrent une préférence pour l'accouplement avec des femelles jeunes et vierges afin de réduire le risque de compétition spermatique. Chez divers escargots terrestres à hermaphroditisme simultané, la production de spermatozoïdes précède la production des œufs de 2–4 semaines, ce qui résulte en une période d'hermaphroditisme protandrique avant la fin de la croissance de la coquille. Nous avons prélevé dans une population naturelle des paires accouplées de l'escargot terrestre à hermaphroditisme simultané *Arianta arbustorum* (L., 1758) comprenant ou bien deux adultes, ou deux subadultes ou alors un adulte et un subadulte; nous avons ensuite déterminé la paternité des nouveau-nés issus des œufs qui ont été subséquemment déposés. Les escargots adultes utilisent le sperme reçu de leurs partenaires d'accouplement subadultes à la même fréquence que le sperme provenant des adultes, ce qui indique qu'il n'y a pas de différence dans la fonction mâle entre les subadultes et les adultes. De plus, une coquille incomplète n'est pas un indicateur fiable de virginité, puisque 35 % des individus subadultes possèdent déjà du sperme accumulé à partir d'un ou plusieurs d'accouplements antérieurs. Par rapport aux adultes, les jeunes affichent un risque plus faible de compétition spermatique, ce qui est indiqué par une préséance plus forte du sperme du dernier partenaire. Cependant, les escargots subadultes produisent moins d'œufs que les adultes, ce qui contrecarre l'avantage évolutif qu'il y a à préférer un partenaire jeune à faible risque de compétition spermatique.

[Traduit par la Rédaction]

Introduction

Sexual selection in animals may act before or after copulation. Precopulatory mechanisms include male competition and female mate choice (Birkhead and Møller 1998), whereas sperm competition and cryptic female choice are considered postcopulatory mechanisms (Birkhead and Pizzari 2002). Sperm competition arises when fertile sperm from two or more males co-occur within a female reproductive tract and compete to fertilize her ova (Parker 1970). The resulting evolutionary arms race has led to a variety of behavioural, morphological, and physiological adaptations, which either enhance the competitive advantage of a male's sperm or counter the sperm of competitors (Birkhead and Møller 1998; Birkhead et al. 2009). For males, one possible mating strategy is to copulate with females that exhibit a low risk of sperm competition. Theory of mate selection predicts that males should prefer to mate with nonexperienced (virgin) females because virgins do not yet store sperm from other males, which may compete for fertilization success (Parker 1998). Thus, males mating with virgin females might have a higher reproductive success and higher contribution in the population asset than males mating with females already storing sperm from previous mate(s). However, this prediction is based on the assumption that males are able to recognize the mating status of the potential partner. In a variety of animal species, chemical and (or) age-depending cues have been re-

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ported as indicators for the mating status of potential partners (Rutowski 1982, Simmons et al. 1994). There is evidence from various gonochoristic species in which males show a preference for mating with virgin or young females to reduce sperm competition risk for their own sperm and thus to increase siring success (Simmons et al. 1994; Bateman and Ferguson 2004). In other species, however, males are not able to recognize the mating status of females (Elgar et al. 2003; Koene et al. 2008). Similar information is so far not available for any species of terrestrial simultaneous hermaphrodites.

Simultaneous hermaphrodites are functional female and male at the same time. Thus, precopulatory sexual selection processes are influenced by both the female and the male traits of an individual. During simultaneous intromission and sperm exchange, a sperm donor is at the same time a sperm receiver. Consequently, a sexual conflict between female and male interests may arise within an individual (Michiels 1998).

Research on mating strategies in simultaneous hermaphrodite land snails has been hampered by the notorious difficulty with which mating can be reliably observed in natural populations. Evidence for promiscuity and multiple paternity in broods is available for several snail species. Individuals of *Helix pomatia* (L., 1758), *Cornu aspersum* (Müller 1774), *Cepaea nemoralis* (L., 1758), and *Arianta arbustorum* (L., 1758) have been observed to mate repeatedly with different partners in the course of a reproductive season resulting in multiple-sired broods (Wolda 1963; Baur 1994*a*; Lind 1988; Rogers and Chase 2002; Evanno et al. 2005). However, little attention has been devoted to the fitness consequences of different mating strategies in land snails (Jordaens et al. 2007).

In the present study, we used microsatellite markers to investigate sperm utilization patterns in young (nonexperienced, subadult) and adult (experienced) individuals of the land snail *A. arbustorum* mating in the wild. This simultaneously hermaphroditic snail has a determinate growth. Sexual maturity is attained at an age of 2–4 years when shell growth is completed (Baur and Raboud 1988). However, sperm production precedes egg production by 2–4 weeks, resulting in a short period of protandric hermaphroditism in this otherwise simultaneous hermaphrodite (Luchtel et al. 1997). Matings involving individuals that had not yet finished shell growth have been observed in natural populations (Baur 1984). Mate choice experiments revealed an absence of precopulatory discrimination between individuals of different size, relatedness, and mating experience (Baur 1992; Baur and Baur 1997; E. Häussler, unpublished data). *Arianta arbustorum* mates repeatedly in the course of a reproductive season and fertile sperm can be stored for more than 1 year in the sperm storage organ (Baur 1988*a*). High levels of multiple paternity have been recorded in the wild (Kupfernagel et al. 2010). It is, however, unknown whether copulating subadult snails with unfinished shells already function as simultaneous hermaphrodites, i.e., whether they deliver fertile autosperm and store the allosperm received for later fertilization of their eggs. Furthermore, it is not known whether adult snails primarily mate with subadult individuals to deliver autosperm rather than to receive allosperm.

We collected copulating pairs of *A. arbustorum* consisting either of two fully grown individuals, of two snails with unfinished shells, or of one fully grown and one snail with unfinished shell in a natural population. After copulation, the snails were kept singly under laboratory conditions, where they deposited eggs. The mothers, their offspring, and the (last) mating partner were genotyped using microsatellite DNA markers. The patterns of paternity were reconstructed and the proportion of offspring sired by the last known mate was assessed. In particular, we addressed the following two questions. (1) Do adult snails show a preference for sperm from adult (experienced) mating partners and consequently use less frequently sperm from subadult snails for the fertilization of their eggs? (2) Does an unfinished shell indicate a virgin mating partner with a low risk of sperm competition for an adult snail?

Materials and methods

Study organism

Arianta arbustorum is common in moist habitats of northwestern and central Europe (Kerney and Cameron 1979). The snail has determinate growth (shell breadth of adults 17–22 mm). Mating in *A. arbustorum* includes elaborate courtship behaviour, which lasts 2–18 h (Baur and Baur 1992*b*); both snails transfer simultaneously one spermatophore. Snails deposit one to three clutches consisting of 20–50 eggs per year (Baur 1990). Cross-fertilization is the dominant mode of reproduction in *A. arbustorum*, though in the absence of a potential mating partner, self-fertilization was observed in snails kept isolated for 2–3 years. However, these snails produced a reduced number of eggs (1%–2% of mated snails) with a decreased hatching success (Chen 1994). In natural populations of *A. arbustorum*, genetic analyses revealed a low frequency of self-fertilizations in one population, but no selfing in three other populations (Kupfernagel et al. 2010). High levels of multiple paternity were recorded in offspring of mother snails sampled in the wild with contributions of 2–6 fathers (Kupfernagel et al. 2010). A controlled laboratory experiment showed that one successful copulation per reproductive season is sufficient to fertilize all the eggs produced by one individual (Chen and Baur 1993). There is, however, a probability of 5%–8% that a copulation does not lead to fertilization of eggs owing to lack of sperm transfer, transfer of infertile sperm, or sperm digestion (Chen and Baur 1993).

Snail samples

Copulating pairs consisting either of two fully grown individuals ($N = 8$ pairs), of two snails that had not yet completed shell growth ($N = 8$ pairs), or of one fully grown and one snail that had not yet completed shell growth ($N = 5$ pairs) were collected in the subalpine forest near Gurnigelbad, 30 km south of Bern, Switzerland (46°45′N, 7°27.5′E; elevation 1250 m above sea level), on three occasions (on 9 and 16 May 2009 and on 2 May 2010). For simplicity, fully-grown adult individuals that had already reproduced in the preceding year(s) are referred to as adult snails and individuals that had not yet finished shell growth when copulating (and thus were assumed to be virgins) are called subadult snails. The sampling dates were 2–4 weeks after the arousal from hibernation. In this forest, *A. arbustorum* is abundant in small clearings with wet soil, along brooks, and in the em-

bankments of tracks constituting a kind of metapopulation (Akçakaya and Baur 1996).

After mating, the snails were kept isolated in transparent beakers (8 cm depth, 6.5 cm diameter) lined with moist soil (approximately 4 cm) at 19 °C under a 16 h light : 8 h dark cycle. Fresh lettuce was provided twice a week and at the same time the beakers were cleaned. The beakers were checked for eggs once per week. The eggs of each clutch were collected, counted, and kept in a plastic dish (6.5 cm diameter) lined with moist paper towels at 19 °C to determine hatching success. Newly hatched snails were separated from remaining unhatched eggs to prevent egg cannibalism (Baur 1994*b*). Hatchlings labelled in order of emergence were stored at –80 °C. Eggs were collected over a period of 60 days. The length of this period corresponds approximately to one reproductive season of *A. arbustorum* living in subalpine forests (Baur 1990).

The shell breadth of each mother snail was measured to the nearest 0.1 mm immediately after sampling using vernier callipers. Subadult individuals completed shell growth by building a reflected lip at the shell aperture within 1–3 weeks.

Table 1. Summary statistics for four microsatellite loci used in paternity analyses of the land snail *Arianta arbustorum* for 2009 and in 2010.

Locus and year	*N*	H_o	H_e	Exclusion probability*
24				
2009	7	0.70	0.71	0.54
2010	8	0.83	0.76	0.49
26				
2009	6	0.74	0.72	0.49
2010	6	0.67	0.73	0.59
55				
2009	6	0.83	0.66	0.49
2010	4	0.61	0.60	0.33
A9				
2009	9	0.87	0.85	0.63
2010	6	1	0.83	0.54

Note: Data are based on 24 mother snails (sampling in 2009) and 18 mother snails (sampling in 2010). *N*, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity.

*Range of combined exclusion probabilities for all loci were 0.83–0.95 for 2009 and 0.83–0.93 for 2010.

Paternity analyses

DNA of individual hatchlings and of mother snails (20–30 mg foot tissue) was extracted following the DNeasy protocol of Qiagen AG (2006). We screened the DNA of mother snails of this population for microsatellite repeats using the nine primer pairs developed by Armbruster et al. (2005) and tested in a previous study (Kupfernagel et al. 2010). Primers that did not amplify or produced only a small number of alleles were not considered. Four highly variable microsatellite loci (*24*, *26*, *55*, and *A9*; Table 1) were employed for paternity analyses. Polymerase chain reaction (PCR) mixtures (HotStarTaq Mastermix Kit; Qiagen AG, Hombrechtikon, Switzerland) were 4–6 ng of DNA (3 µL) in a total volume of 10 µL following the protocol of Qiagen AG (2005). PCR mixtures were preheated at 95 °C for 15 min, followed by 30–35 cycles of 95 °C for 30 s, locus-specific annealing for 30 s, and 72 °C for 30 s (Armbruster et al. 2005). PCR was finished with an extension of 8 min at 72 °C. Horizontal electrophoresis was performed with SEA2000™ advanced submerged gel electrophoresis equipment. Spreadex® EL400-gels (Elchrom Scientific AG, Cham, Switzerland) were used.

We genotyped 1204 hatchlings from 41 mother snails that were engaged in 21 copulations (one subadult mother snail did not produce any eggs). We analyzed 50 hatchlings from each adult mother snail ($N = 21$). Within family, hatchlings were randomly chosen from the first and second clutch laid (Table 2). Subadult mother snails were expected to be virgin prior to the observed copulation and consequently their offspring should be sired by a single father snail. We therefore genotyped only 10 hatchlings from each subadult mother snail ($N = 20$) to confirm single paternity and to test for self-fertilization.

Null alleles can significantly affect estimates of genetic relatedness of individuals and population genetic structure (Pemberton et al. 1995; Dakin and Avise 2004). Null alleles as a result of low DNA concentration or PCR failure were detected in 7 (0.6%) out of the 1204 hatchlings examined. Data from these seven hatchlings were omitted. Thus, in the paternity analysis, a total of 1197 hatchlings were considered. This corresponds to 45.4% of all hatchlings produced by the 41 mother snails (subadult mothers: 19.9%; adult mothers: 65.5%).

Data analyses

Microsatellite characteristics were assessed using both GenAlEx version 6.1 (Peakall and Smouse 2006), a Microsoft Excel add-on developed for population genetic data analyses, and GENEPOP (Raymond and Rousset 1995; available from http://genepop.curtin.edu.au, accessed DAY MONTH YEAR). Statistical power of paternity analysis was assessed by calculating the probability of detecting multiple paternity using PrDM (Neff and Pitcher 2002).

Sibship analysis and parentage reconstruction of the field-collected mother snails and their progeny were performed with GERUD version 2.0 (Jones 2005) and COLONY version 1.2 (Wang 2004) to estimate the number and genotypes of the contributing fathers in the progeny. GERUD uses multiple-locus data for reconstruction of the contributing paternal genotype(s) from mother to progeny arrays. The program does not distinguish between potential fathers of the same genotype. Consequently, the number of paternal genotypes estimated is equal to a minimum number of involved fathers. The maximum likelihood program COLONY (Wang 2004) was used to assess the genotypes of fathers and the total number of fathers contributing their gametes to a progeny array. Considering the different allele frequencies in the copulating mother snails sampled either in 2009 or in 2010, we conducted the paternity analysis separately for each year with the software COLONY. For the paternity assignment, the maternal genotypes of the population were used. The maternal genotypes represent also the potential paternal genotypes in this simultaneous hermaphrodite. COLONY provides the most probable configuration of paternity, including assignments of every offspring to one of the estimated paternal genotypes. This allows us to assign the offspring to the last and to previous mating partner(s) in the wild.

Table 2. Estimated number of fathers contributing to the offspring of mothers of the land snail *Arianta arbustorum* collected in the wild, and variation in multiple paternity in successive clutches.

Mother ID	Growth status*	Last mating partner ID	Last mate status*	Sampling date†	Hatchlings analysed (1st clutch; 2nd clutch)	Minimum number of fathers‡	Total number of fathers§	K_e‖	P_L¶ (1st clutch; 2nd clutch)	Distribution of fathers in all genotyped hatchlings**
1	A	2	A	1	25; 25	3	6	4.72	0.08 (0.08; 0.08)	**4a**,12b,13c,10d,1e,10f
2	A	1	A	1	20; 14	5	6	3.42	0.47 (0.70; 0.14)	**16a**,7b,2c,4d,2e,3f
3	A	4	A	1	25; 25	5	8	6.79	0.12 (0.20; 0.04)	**6a**,7b,10c,3d,10e,7f,3 g,4h
4	A	3	A	1	25; 25	5	9	6.19	0.24 (0.28; 0.20)	**12a**,11b,2c,3d,6e,3f,4 g,8h,1i
5	A	6	A	2	25; 25	4	6	3.85	0.04 (0.04; 0.04)	19a,14b,3c,**2d**,4e,8f
6	A	5	A	2	25; 25	5	9	6.94	0.04 (0.04; 0.04)	4a,13b,4c,5d,8e,5f,**2 g**,5h,4i
7	A	8	A	2	25; 25	4	7††	5.51	0.08 (0.16; 0)	11a,12b,**4c**,6d,10e,6f,**1g**
8	A	7	A	2	25; 25	4	4	2.21	0.02 (0.04; 0)	31a,7b,**1c**,11d
53	A	54	A	3	25; 6	3	5	4.51	0.16 (0.20; 0)	**5a**,10b,6c,4d,6e
54	A	53	A	3	21; 25	3	5	4.32	0.13 (0.29; 0)	13a,14b,**6c**,8d,5e
55	A	56	A	3	24; 25	4	7	5.10	0.24 (0.08; 0.40)	5a,14b,4c,5d,8e,**12f**,1 g
56	A	55	A	3	25; 25	2	3	2.76	0.46 (0.40; 0.52)	11a,**23b**,16c
57	A	58	A	3	23; 25	5	8	6.51	0.15 (0.17; 0.12)	7a,3b,8c,6d,11e,**7f**,1 g,5h
58	A	57	A	3	25; 24	2	5	4.16	0.12 (0.16; 0.08)	10a,18b,**6c**,9d,6e
59	A	60	A	3	25; 25	2	4	2.07	0.02 (0.04; 0)	4a,32b,13c,**1d**
60	A	59	A	3	25; 25	3	6	4.33	0.04 (0.04; 0.04)	7a,14b,16c,3d,**2e**,8f
9	A	111	S	2	19; 25	3	6††	5.04	0.20 (0.37; 0.08)	5a,**9b**,13c,6d,3e,*8f*
10	A	112	S	2	25; 25	4	6	5.04	0.16 (0.16; 0.16)	5a,15b,**8c**,11d,6e,5f
11	A	113	S	2	25; 24	3	4	3.09	0.08 (0.16; 0)	22a,14b,**4c**,9d
51	A	157	S	3	25; 23	3	9	6.40	0.21 (0; 0.43)	**10a**,5b,1c,10d,8e,7f,4 g,1h,2i
52	A	158	S	3	25; 25	3	5	3.81	0.38 (0.19; 0.58)	**19a**,11b,7c,11d,2e
101	S	102	S	1	5; 5	1	1	1.00	1 (1; 1)	**10a**
102	S	101	S	1	5; 5	1	1	1.00	1 (1; 1)	**10a**
103	S	104	S	1	5; 5	1	1	1.00	1 (1; 1)	**10a**
104	S	103	S	1	10; 0	2	2	1.22	0.90 (0.90; —)	**9a**,1b
105	S	106	S	2	5; 5	1	1	1.00	1 (1; 1)	**10a**
106	S	105	S	2	5; 5	1	1	1.00	1 (1; 1)	**10a**
107	S	108	S	2	5; 5	1	1	1.00	1 (1; 1)	**10a**
108	S	107	S	2	5; 5	1	3	2.38	0.10 (0; 0.20)	5a,4b,**1c**
109	S	110	S	2	5; 5	1	1	1.00	1 (1; 1)	**10a**
110	S	109	S	2	5; 5	2	2	1.72	0.70 (0.40; 1)	3a,**7b**
151	S	152	S	3	5; 5	2	2	1.22	0.90 (0.80; 1)	**9a**,1b
152	S	151	S	3	5; 5	1	1	1.00	1 (1; 1)	**10a**
153	S	154	S	3	5; 5	1	1	1.00	1 (1; 1)	**10a**
154	S	153	S	3	5; 4	1	1	1.00	1 (1; 1)	**9a**
155	S	156	S	3	5; 5	3	3	1.85	0.70 (0.80; 0.60)	**7a**,2b,1c
156	S	155	S	3	5; 5	2	2	1.92	0.40 (0.20; 0.60)	**4a**,6b
111	S	9	A	2	5; 5	1	1	1.00	1 (1; 1)	**10a**
112	S	10	A	2	5; 5	1	1	1.00	1 (1; 1)	**10a**
113	S	11	A	2	0	—	—	—	—	—

Table 2 (*concluded*).

Mother ID	Growth status*	Last mating partner ID	Last mate status*	Sampling date†	Hatchlings analysed (1st clutch; 2nd clutch)	Minimum number of fathers‡	Total number of fathers§	K_e‖	P_L¶ (1st clutch; 2nd clutch)	Distribution of fathers in all genotyped hatchlings**
157	S	51	A	3	5; 5	1	2	1.47	0.80 (0.60; 1)	2a,**8b**
158	S	52	A	3	5; 5	1	1	1.00	1 (1; 1)	**10a**

*A, adult; S, subadult.
†(1) 9 May 2009; (2) 16 May 2009; (3) 2 May 2010.
‡Parentage reconstruction using GERUD.
§Likelihood assessment based on genetic diversity using COLONY.
‖Effective paternity estimates.
¶Proportion of hatchlings sired by the last of several mates.
**Genotyped father (last copulation in boldface type.
††Occurrence of self-fertilization.

We also calculated the effective paternity index (K_e) following Johnson and Yund (2007). This index depends on the relative skew among paternity shares and is maximized when shares are equal. Differential sperm usage from two consecutive matings for the fertilization of eggs of a single female is usually expressed by P_2 (the proportion of eggs fertilized by the second of two mates; Boorman and Parker 1976). Analogously, we calculated sperm precedence of the last of several consecutive mates, P_L, as the proportion of hatchlings sired by the last of several mates. The genotype of the last mate was known because copulating pairs of snails were collected in the field.

Differences in level of multiple paternity and last mate sperm precedence were analysed using the Kruskal–Wallis test followed by the Mann–Whitney *U* test for group comparisons. We applied ANCOVAs with the factors growth stage of the mother and age of the last mating partner and the cofactor shell size to examine possible effects on the number of eggs produced (log-transformed) and hatching success (arcsine-transformed). The rate of self-fertilization was quantified using genotyped data of the offspring and their parents. Statistical analyses were conducted using PASW® statistics 18.0 core system (SPSS Inc. 2009), unless otherwise noted. Values are reported as mean ± SD, unless otherwise noted.

Results

Microsatellite variation

We considered the 42 mother snails as reference sample of the population. The four loci used in this study varied in the level of polymorphism from four to nine alleles (Table 1). Genotype frequencies at all loci were within expectations of Hardy–Weinberg equilibrium ($P > 0.05$), and no evidence of genotypic disequilibrium between pairs of loci was found ($P > 0.05$). Paternity exclusion probability for all four loci combined was 0.95 to 0.99 (GenAlEx estimates). Probabilities of detecting multiple paternity were ≥96% for all clutches when assuming equal paternal contributions of the involved fathers, and ≥88% when assuming skewed paternal contribution.

Level of multiple paternity

Considering adult individuals, multiple paternity was found in the offspring of all mother snails (Table 2). The level of multiple paternity, however, was highly variable among mother snails. The most conservative paternity estimate, the minimum number of fathers, obtained from all loci with GERUD version 2.0, was in the range of 2 to 5 (3.6 ± 1.0; Table 2). The estimates of most likely numbers of fathers from COLONY ranged from 3 to 9 contributing males per mother snail with a mean of 6.1 (SD = 1.76; Table 2). The estimates of contributing fathers by the two methods matched relatively well (Spearman rank correlation, $r_S = 0.69$, $N = 21$, $P = 0.001$), considering that the first method estimates only the minimum number of fathers, whereas the most probable number of sires given by the second method should be higher than the minimum (Table 2). In further analyses, estimates of the second method (COLONY) are only considered.

The extent of multiple paternity was influenced neither by the shell volume of the mother snail (Spearman rank correlation, $r_S = –0.01$, $N = 21$, $P = 0.99$) nor by the sampling date

(Kruskal–Wallis test, $\chi^2_{[2]} = 2.48$, $P = 0.29$). Furthermore, the mating experience of the last mate did not influence the level of multiple paternity in adult mother snails (Mann–Whitney U test; A×A vs. A×S: $z = -0.21$, $P = 0.83$).

In contrast to our expectations, not all of the subadult snails copulated for the first time when they were collected, as indicated by the multiple paternity in their offspring (Table 2). Multiple paternity was found in the progeny of 7 out of 20 (35.0%) subadult snails. In these 7 subadult mother snails, the minimum and total number of fathers, obtained from all loci with COLONY ranged from 2 to 3 (2.3 ± 0.5). Subadult snails with multiple paternity did not differ in shell size from subadult snails with single paternity (Mann–Whitney U test, $z = 0.87$, $N = 20$, $P = 0.38$). Furthermore, the proportion of subadult mother snails with multiple paternity in their offspring did not differ between the three sampling dates ($\chi^2_{[2]} = 1.32$, $P = 0.52$).

Low frequencies of self-fertilization were found in 2 out of 41 mother–progeny arrays: 2.0% of the offspring of an adult mother snail that had copulated with an adult partner and 18.2% of the offspring of an adult snail that had copulated with a subadult partner (Table 2).

Patterns of sperm utilization

Patterns of sperm utilization were examined in the progeny of mother snails with multiple paternity (adults: $N = 21$; subadults: $N = 7$). Different fathers sired different numbers of offspring in the progeny of 13 (61.9%) adult and of 3 (42.9%) subadult mother snails (contingency test, in all 16 cases $P < 0.05$). The different fathers sired equal numbers of offspring ($P > 0.05$) in the progeny of the remaining 12 mother snails (8 adults and 4 subadults) with observed multiple paternity. The effective paternity estimate (K_e) ranged from 2.1 to 7.0 in adult mother snails (4.6 ± 1.4) and from 1.2 to 2.4 (1.7 ± 0.4) in subadult mother snails (Table 2). K_e estimates correlated with multiple paternity estimates of GERUD and COLONY (Spearman rank correlation, GERUD: $r_S = 0.74$, $N = 28$, $P < 0.0001$; COLONY: $r_S = 0.95$, $N = 28$, $P < 0.0001$).

Last mate sperm precedence (P_L) ranged from 0.02 to 0.47 (0.16 ± 0.13, $N = 21$) in adult mother snails and from 0.10 to 0.90 (0.64 ± 0.29, $N = 7$) in subadult mother snails with multiple copulations. P_L was significantly higher in subadult mother snails with multiple matings than in adult mother snails (Mann–Whitney U test: $z = 3.11$, $N = 28$, $P = 0.002$). In adult mother snails, the growth status of the last mating partner (adult or subadult) did not influence last mate sperm precedence (means: 0.15 vs. 0.20; Mann–Whitney U test: $z = 1.29$, $N = 21$, $P = 0.20$). This suggests that adult individuals use sperm received from subadult snails for the fertilization of their eggs as frequently as sperm from adult snails. Considering all snails, P_L values were negatively correlated with the level of multiple paternity observed in the progeny–mother arrays (Spearman rank correlation, $r_S = -0.42$, $N = 28$, $P = 0.03$).

Female reproductive output

The number of eggs produced by a mother snail was influenced by the growth status of the individual (adults: 80.0 ± 19.0, $N = 21$; subadults: 69.9 ± 22.5, $N = 20$; ANCOVA: $F_{[1,36]} = 4.88$, $P = 0.03$) and the size of the mother (adults: 1.40 ± 0.39 cm^3, $N = 21$; subadults: 1.31 ± 0.28 cm^3, $N = 20$; $F_{[1,36]} = 4.44$, $P = 0.04$) but not by the growth status of the last mating partner ($F_{[1,36]} = 1.00$, $P = 0.32$). None of the interactions, i.e., between growth status of the mother snail and growth status of the last mating partner, was significant. Hatching success of eggs averaged 89.1% (SD = 12.5%, range 50%–100%, $N = 41$) and was influenced neither by the age of the mother nor by the age of the last mating partner or the size of the mother (ANCOVA; growth status of mother: $F_{[1,36]} = 1.25$, $P = 0.27$; growth status of last mate: $F_{[1,36]} = 2.17$, $P = 0.15$; mother size: $F_{[1,36]} = 0.01$, $P = 0.93$).

The genotyping of offspring allows also an estimate of the number of hatchlings sired by each snail as a result of the last copulation observed in the field. Consequently, the number of hatchlings produced (a measure of female fitness) and the proportion of hatchlings sired following the last copulation by the last mating partner (a measure of male fitness) can be assessed for each snail. Most interestingly, the number of hatchlings produced of each mother snail was negatively correlated with the proportion of hatchlings sired in the last mating partner (Spearman rank correlation, $r_S = -0.34$, $N = 41$, $P = 0.03$). However, considering only mother snails with multiple paternity or fully grown mother snails, this correlation was no longer significant (Spearman rank correlation, $r_S = -0.25$, $N = 28$, $P = 0.19$ and $r_S = -0.37$, $N = 21$, $P = 0.09$, respectively).

Discussion

The present study showed that adult snails used sperm received from subadult mating partners for the fertilization of their eggs in the same frequency as sperm from adult mating partners. This indicates that the male function of snails with unfinished shells do not differ that from adult snails. Furthermore, an unfinished shell cannot be considered a reliable indicator for virginity, because 35% of the subadult snails had already successfully mated prior to the copulation observed in our study.

Mate selection aims at differences in morphological characters, but also at differences in genetic and fitness traits (Andersson 1994). Mate choice and sexual strategies evolved to gain the most effective reproductive success, i.e., a high contribution of fit offspring to the population asset. According to the theory of mate selection, males should prefer to mate with virgin (subadult) females to accomplish a higher siring success (Birkhead and Møller 1998). Owing to sperm storage from previous copulations, mating with a nonvirgin partner may result in a higher risk of sperm competition than mating with a virgin partner (Parker 1998). In insects, mate choice experiments revealed a preference of males for copulating with young or virgin females (Simmons et al. 1994; Bateman and Ferguson 2004). In a variety of gonochoristic invertebrate species, growth and size are important indicators for the age and mating history of the potential partner (Rutowski 1982; Simmons 1995). It has been suggested that precopulatory sexual selection on traits related to mate acquisition is intrinsically weaker in simultaneously hermaphrodites than in gonochoristic animals (Greeff and Michiels 1999). Consequently, postcopulatory selection might be

more essential in simultaneous hermaphrodites, particular in the presence of long-term sperm storage, complex sperm storage organs, and mechanisms for digestion of excess sperm (Baur 1994*c*, 1998).

Although body size is a good indicator of female reproductive output in terms of eggs in *A. arbustorum* (Baur 1988*b*), this does not result in a preference for mating with large partners (Baur 1992). Random mating with respect to size is common in hermaphroditic gastropods (*Aplysia californica* J. G. Cooper, 1863: Pennings 1991; *Lymnaea stagnalis* (L., 1758): Koene et al. 2007; *Cepaea nemoralis*: Wolda 1963; *Helix pomatia*: Baur 1992). Exceptions from random mating can be explained by physical constraints or extended protandric periods. In the nudibranch *Chromodoris zebra* (Heilprin, 1888) (= *Hypselodoris zebra* (Heilprin, 1889)), sexually mature slugs range in body length from 4 to 18 cm and two individuals that differ greatly in size are unable to bring their reproductive organs together resulting in size-assortive mating (Crozier 1918). The terrestrial pulmonate *Achatina fulica* (Férussac, 1821) is a protandrous hermaphrodite with indeterminate growth. Subadult snails, which produce only sperm, continue to grow for an additional 3–6 months to become true hermaphrodites (Tomiyama 1993). In a natural population with 72% protandric and 28% hermaphroditic snails in Japan, copulations between hermaphroditic individuals occurred more frequently than would be expected under random mating (Tomiyama 1996).

Matings between subadult and adult individuals have been observed in several hermaphroditic gastropod species including *Theba pisana* (Müller, 1774) (Cowie 1980), *A. arbustorum* (Baur 1984), and the nudibranch *Phestilla sibogae* Bergh, 1905 (Todd et al. 1997). Mate choice experiments revealed that snails do not discriminate between subadult and adult partners in *A. arbustorum* (E. Häussler, unpublished data). Furthermore, individuals of *A. arbustorum* are not able to adjust sperm expenditure to the mating history of the partner (Baur et al. 1998). In the hermaphroditic pond snail *L. stagnalis*, in contrast, the number of sperm transferred is influenced by the mating history (Koene et al. 2008). Focal snails delivered more sperm to individuals previously kept in isolation (virgins) than to those kept in groups (nonvirgins) (Loose and Koene 2008). Allohormones, which affect sperm utilization of the mating partner in *L. stagnalis* (Koene 2005), may influence sperm delivery independent of a recognition of the mating status in this species (Koene et al. 2010).

The random mating pattern does not imply random fertilization of eggs, because multiple mating and sperm storage offer opportunities for sperm competition (Baur 1998). Furthermore, the structure and morphology of the sperm storage site (spermatheca), fertilization chamber, and sperm digestion organ of *A. arbustorum* offer opportunities for sperm selection by the female function of the hermaphrodite (cryptic female choice; Haase and Baur 1995). The lack of mate choice with respect to mating history does not imply that there is no precopulatory mate choice at all in *A. arbustorum*. Choice experiments revealed precopulatory discrimination between individuals from two distant populations of *A. arbustorum* in Switzerland, suggesting incipient reproductive isolation (Baur and Baur 1992*a*; Kupfernagel and Baur 2011). It is assumed that individuals may use population-specific cues (pheromones, behaviour) during courtship and copulation to assess the affiliation of potential mates.

In natural populations of *A. arbustorum*, high levels of multiple paternity have been recorded in single egg clutches and a low frequency of self-fertilization in a few mother snails (Kupfernagel et al. 2010). In the present study, we found a similar level of multiple paternity and a low frequency of self-fertilization in adult *A. arbustorum* as previously recorded in wild-caught individuals (Kupfernagel et al. 2010). In both studies, the level of multiple paternity was not influenced by shell size, which could be explained by a lack of size-assortive mating in this species (Baur 1992).

More than a third (35%) of the subadult snails had stored sperm from previous mating(s) and thus were nonvirgins during the copulation observed in the field. This indicates that an unfinished shell in *A. arbustorum* is not a reliable indicator for a virgin mating status. Most interestingly, however, last sperm precedence in subadult mother snails with multiple paternity was higher than in adult snails (0.64 vs. 0.16). This suggests that it may be beneficial for any snail to mate with a subadult partner, irrespectively whether or not it is virgin. On the other hand, subadult snails produced fewer eggs than adults in our study, most probably because they still had to invest resources into growth. Consequently, the reduced number of offspring produced may counteract the evolutionary advantage of preferring a young partner with low sperm competition risk.

In the present study, adult snails utilized sperm received from subadult partners in similar frequencies for the fertilization of their eggs as sperm from adult partners. This indicates that sperm utilization of the focal snail is not influenced by the mating history and growth stage of its partner. The lack of precopulatory mate choice in *A. arbustorum* could be explained in different ways. It could be a result of the particular biology of the species. In species with low active dispersal like land snails, encounters with potential mates may be rare in low-density populations. Furthermore, sperm depletion over time, most pronounced after hibernation (Baur 1994*c*), may affect the reproductive success. Moreover, courtship and copulation in terrestrial gastropods are restricted to periods of favourable environmental conditions. It has been suggested that because of time-constrained activity and high costs for locomotion, the best strategy for a snail is to mate with the first mating partner available to minimize the risk of either ending up without any mating at all or drying up during mating (Baur 1992).

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Chapter 4

Partial precopulatory isolation between two geographically distant populations of the land snail *Arianta arbustorum* (L.)

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PARTIAL PRECOPULATORY ISOLATION BETWEEN TWO GEOGRAPHICALLY DISTANT POPULATIONS OF THE LAND SNAIL *ARIANTA ARBUSTORUM* (L.)

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ABSTRACT

Different mechanisms of reproductive isolation were examined in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Snails from two geographically isolated populations in the Swiss Alps were allowed to copulate with both a homotypic (individual from the same population) and a heterotypic (individual from the other population) partner (in half of the pairs in reversed order). Control snails mated twice with a homotypic partner. In the first mating, successful copulations occurred in a lower frequency in heterotypic pairs (55.6%) than in homotypic pairs (82.9%). Heterotypic pairs that eventually copulated showed more breaks during courtship than homotypic pairs. However, neither the number of eggs produced nor their hatching success was influenced by the type of mating partner. In the second mating, the sequence of different partners had an effect on the proportion of successful copulations in snails from one geographical population. Snails that copulated first with a homotypic partner remated more frequently with a homotypic partner than snails that copulated first with a heterotypic partner. Paternity analyses of progeny of snails that mated twice indicate no influence of the origin of the mating partner. The proportion of hatchlings sired by the second mate (P_2) averaged 0.39, indicating a slight first-mate advantage. However, highly skewed paternity patterns were found in the progeny of 44.4% of the double-mated snails. Genetic analyses also revealed a low frequency of self-fertilization (3.7%). These findings indicate the presence of partial precopulatory isolation between two distant snail populations, although reproductive compatibility is still maintained.

INTRODUCTION

Reproductive isolation is an important step in the process of speciation. In animals, reproductive isolation mechanisms can appear before or after copulation and are therefore divided into precopulatory and postcopulatory isolation mechanisms (Mayr, 1963). Reproductive barriers can take various forms and can be established in different ways, for example, by geographical separation of small populations or changes in behavioural patterns (Coyne & Orr, 2004; Rundle & Nosil, 2005). Such shifts seldom evolve spontaneously, but often gradually over generations (Barton & Charlesworth, 1984).

The occurrence of reproductive isolation mechanisms between natural populations has an important impact on the reduction of gene flow and can therefore influence the genetic diversity and fitness of populations. Moreover, inbreeding, i.e. mating between close relatives and, in hermaphrodites, self-fertilization (Jarne & Auld, 2006), and outbreeding, i.e. reproduction between unrelated individuals from different populations (heterotypic), influence genetic diversity and fitness in natural populations (Frankham, 1995).

In recent years, the impact of inbreeding has been investigated extensively in gonochoristic (species with separate sexes) and in hermaphroditic animals. In contrast, the effects of outbreeding have been given little attention, especially in hermaphroditic animals (for an exception see McCarthy & Sih, 2008). However, outbreeding depression, where offspring from heterotypic copulations show a reduced fitness and lower adaptation ability to prevailing conditions than offspring from local (homotypic) copulations, can be seen as one component for evolving reproductive isolation.

Hermaphroditic land snails with low dispersal ability are excellent study organisms for the examination of isolation mechanisms evolving in natural populations (Gittenberger, 1988; Baur & Baur, 1992a; Schilthuizen *et al*., 2006). Different localities are characterized by different selection pressures and, with low dispersal capacity and limited gene flow, small snail populations adapt differently from locality to locality (Hanski & Gilpin, 1997). Reproductive isolation mechanisms can arise gradually and outbreeding effects between different localities can be tested. Additionally, in populations with reduced gene flow, the selection of specific partner traits can lead to the development of population-specific partner preferences, which can increase the reproductive isolation of a population. As a result, sexual selection processes go hand-in-hand with isolation processes.

Sexual selection, in general, is divided into processes that occur before mating (precopulatory selection, e.g. courtship) and inside the female organs after the mating (postcopulatory selection). Postcopulatory sexual selection can be caused either by sperm competition between different mating partners or by cryptic female choice and selective sperm utilization (Birkhead & Møller, 1998). In simultaneously hermaphroditic animals, precopulatory selection processes are influenced by both the female and the male traits of an individual. Greeff & Michiels (1999) suggested that selection on traits related to mate acquisition is intrinsically weaker in hermaphrodites than in gonochorists and that the postcopulatory mechanism might be more essential. Additionally, in hermaphroditic helicid snails, long-time sperm storage from different mating partners, complex sperm storage organs and a mechanism for the digestion of

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excess sperm are known (Baur, 1998; Beese, Beier & Baur, 2006; Beese *et al*., 2009) and can influence the outcome of the variation observed in sperm utilization (Haase & Baur, 1995; Chase & Darbyson, 2008; Kupfernagel, Rusterholz & Baur, 2010).

In this study, we investigated precopulatory and postcopulatory isolation mechanisms that might have evolved during a long period of geographical isolation in two populations of *Arianta arbustorum* (L.). This simultaneously hermaphroditic land snail has a continuous distribution across northern and western Europe (Kerney & Cameron, 1979). The dispersal of marked individuals averaged 7 m in 1 year (Baur, 1986) and, due to limited dispersal ability, metapopulations exist (Akçakaya & Baur, 1996). Large differences in life-history characters have been reported between distant populations, including shell size, maturation time, egg size and clutch size (Baur, 1984, 1986, 1990; Baur & Gosteli, 1986; Baur & Raboud, 1988).

In hermaphrodites, the ability to reproduce by self-fertilization is a means of coping with limited dispersal ability or low mate-encounter rate in low-density populations (Levins, 1968). Mixed mating systems, where reproduction occurs via self- and cross-fertilization, are common in plants (Goodwillie, Kalisz & Eckert, 2005) and have also been found in a variety of simultaneously hermaphroditic gastropods (Baur, 1987; Heller, 1993; Jarne & Auld, 2006). In general, complete outcrossing is preferred when the inbreeding effects are pronounced (Lande & Schemske, 1985). In natural populations of pulmonate land snails, however, it remains unclear how frequently selfing or outcrossing occur and which mechanisms can lead to a switch in the reproduction mode (Heller, 1993; Backeljau, Baur & Baur, 2001).

Cross-fertilization is the dominant mode of reproduction in *A. arbustorum* although, in the absence of a potential mating partner, self-fertilization can be observed in controlled laboratory experiments (Chen, 1994). However, snails kept isolated for 2–3 years produced a reduced number of eggs (1–2% of mated snails) with a decreased hatching success (Chen, 1994). In natural populations of *A. arbustorum*, genetic analyses revealed a low frequency of self-fertilizations in one population, but no selfing in three other populations (Kupfernagel *et al*., 2010).

The present study was designed to investigate whether selective sperm use and/or self-fertilization is involved in outbreeding avoidance, which may explain patterns of postcopulatory isolation already observed between animals from these geographically distant populations (Baur & Baur, 1992a). Using microsatellite markers, we examined patterns of paternity in mating trials where focal snails mate with both an unrelated snail from the same population and an individual from a geographically remote population in either order. Applying genetic analyses, the mating order effect was analysed by estimating the sperm precedence and the differential sperm utilization from consecutive matings.

MATERIAL AND METHODS

Study organism

Arianta arbustorum is common in moist habitats of northwestern and central Europe (Kerney & Cameron, 1979). The snail has determinate growth (shell breadth of adults 17–22 mm). Individuals become sexually mature at 2–4 years and adults live another 3–4 years (Baur & Raboud, 1988). Mating is random with respect to shell size and different degrees of relatedness (Baur, 1992; Baur & Baur, 1997). Mating behaviour in *A. arbustorum* includes elaborate courtship behaviour, which consists of introductory behaviour (foreplay) with reciprocal tactile and oral contacts, and curving turns to reach an optimal position with respect to the genital opening of the partner (Hofmann, 1923; B.B., unpubl.). This is followed by an optional dart shooting, the pushing of a calcareous dart into the mating partner's body, which is assumed to increase sperm storage in the partner (Rogers & Chase, 2001; Chase 2007). Copulation is reciprocal; both snails simultaneously transfer one spermatophore. The mating process lasts 2–18 h (Baur & Baur, 1992b). After a successful copulation, individuals need at least 8 days to replenish their sperm reserves (Locher & Baur, 1999; Hänggi, Locher & Baur, 2002). *Arianta arbustorum* mates repeatedly in the course of a reproductive season and fertile sperm can be stored for more than 1 year in the sperm storage organ (Baur, 1988). In the field, snails deposit 1–3 egg batches consisting of 20–50 eggs per year (Baur, 1990). A controlled laboratory experiment showed that one successful copulation per reproductive season is sufficient to fertilize all the eggs produced by one individual (Chen & Baur, 1993). There is, however, a probability of 5–8% that a copulation does not lead to fertilization of eggs, due to lack of sperm transfer, transfer of infertile sperm or sperm digestion (Chen & Baur, 1993).

Snail samples

Virgin individuals (subadult snails that had not yet completed shell growth) of *A. arbustorum* were collected in spring 2007 and 2008 from two different sites in the Swiss Alps: in Gantrisch, an alpine pasture 30 km south of Berne (46°42′N, 7°27′E, elevation 1810 m a.s.l.; hereafter referred to as G) and Strela, an alpine pasture with scattered scree 4 km west of Davos (46°49′N, 9°48′E, elevation 2100 m a.s.l.; referred to as S). The sites are situated 180 km apart.

The snails were kept individually in transparent beakers (8 cm deep, 6.5 cm diameter) lined with moist soil (*c*. 4 cm) at 19°C with a light:dark cycle of 16:8 h until they reached sexual maturity as indicated by the formation of a reflected lip at the shell aperture. Fresh lettuce was provided twice a week and at the same time the beakers were cleaned. The snails were marked individually by writing numbers on their shells with a waterproof felt-tipped pen on a spot of correction fluid (Tipp-Ex). The animals showed no visible reaction to the marking procedure.

Mating experiment

The cross-mating experiment was conducted to examine whether premating and/or postmating isolation occurs between individuals from the two distant populations. Prior to mating, sexually mature snails were genotyped using a noninvasive method (see below). Virgin individuals (focal snails) from both populations were mated twice: first with a virgin snail from the same population (homotypic matings) and second with another virgin snail from a distant population (heterotypic matings). In half of the snails the mating order was reversed (Table 1). As a control, focal snails were mated with two virgin partners from the same population. Partners of homotypic matings were not related to each other.

Mating trials were initiated in the evening and run during the night (with natural temperature fluctuation) in the summers of 2007 and 2008, respectively. This period is the time of maximum mating activity in natural snail populations (Cain & Currey, 1968; Wolda & Kreulen, 1973). The focal snail and a randomly chosen sperm donor which, however, differed from the focal snail in at least two highly variable microsatellite primers (see below), were placed in a transparent beaker (measuring 14 × 10 × 7 cm) whose bottom was covered with moistened paper towelling to maintain snail activity. The snails' behaviour was monitored using spot checks at intervals of 20 min from 8 p.m. to 6 a.m. (at night using a flash light).

Table 1. Design of the mating experiment with focal snails of *Arianta arbustorum* that mated twice and type of data collected.

Focal snail (population)	Mating sequence				Mating group*
	First mating partner		Second mating partner		
G	G	a, b, c, –, –	S	a, b, –, d, e	Foc. × Homot. × Heterot.
G	S	a, b, c, –, e	G	a, b, –, d, e	Foc. × Heterot. × Homot.
G	G	a, b, c, –, –	G	a, b, –, d, e	Foc. × Homot. × Homot. (control)
S	S	a, b, c, –, –	G	a, b, –, –, –	Foc. × Homot. × Heterot.
S	G	a, b, c, –, e	S	a, b, –, d, e	Foc. × Heterot. × Homot.
S	S	a, b, c, –, –	S	a, b, –, –, –	Foc. × Homot. × Homot. (control)

Focal snails from two populations (G, Gantrisch; S, Strela) copulated with homotypic or heterotypic partners in different order. Type of data: (a) frequency of successful copulations; (b) mating behaviour; (c) number of eggs produced between first and second copulation; (d) number of eggs produced after second copulation; (e) paternity analysis of offspring.

*In both populations the sequence with two heterotypic partners (Foc. × Heterot. × Heterot.) was not considered.

We recorded the following behaviour: (1) active but no courtship; (2) introductory courtship behaviour, contact between mating partners; (3) progressed courtship, curving turns with swollen genitals; (4) body contact, attempted copulation; and (5) copulation. Records included time until initiation of courtship (courtship latency), courtship duration (time interval from courtship initiation to copulation) and copulation duration. The initiation of courtship was defined as the first simultaneous oral contact (which was usually accompanied by a slight eversion of the penial lobe in at least one of the snails) and the beginning of the copulation as the first simultaneous intromission. Furthermore, we recorded the number and duration of behavioural discontinuities (breaks in mating behaviour). Observation sessions were terminated either when two snails mated or after 10 h if no snail initiated courtship behaviour. Between mating trials, snails were maintained as described above.

After the first copulation, all focal snails were allowed to replenish their autosperm reserves for 7 days (Locher & Baur, 1999). Then, they were allowed to copulate with a second partner following the experimental setup shown in Table 1. Behavioural records were as described in the first copulation. However, most snails did not mate at the first attempt. These individuals were placed together with another potential mate of the same group as the previous one in test boxes after 1 week or more. For each focal snail we also recorded time until first mating, time until second mating and time between second copulation and oviposition. The 106 individuals involved in the first copulation were allowed to remate with a new virgin partner (second copulation).

Reproductive performance

To examine whether individuals that copulated with a partner from a distant population show reduced reproductive performance, we examined the number of eggs and hatchlings produced by each snail. We checked beakers of focal snails for eggs once per week. The eggs of each batch were collected, counted and kept in a plastic dish (6.5 cm in diameter) lined with moist paper towelling at 19°C to determine hatching success. Newly hatched snails were counted and separated from remaining unhatched eggs to prevent egg cannibalism (Baur, 1994b). Labelled in order of emergence, the hatchlings were stored at −80°C until genetic analysis.

Parental genotyping and paternity analyses

DNA of focal snails and mating partners was obtained from foot mucus using a noninvasive technique (Armbruster, Koller & Baur, 2005). We screened the DNA for microsatellite repeats using at least at two highly variable primer pairs (24, 26, 55 or/and A9) developed by Armbruster *et al.* (2005). The same microsatellite loci were considered in paternity analyses using the tissue of hatchlings as DNA source. DNA of focal snails, mating partners and individual hatchlings was extracted following the DNeasy protocol of Qiagen (2006). PCR-mixture (HotStarTaq Mastermix Kit, Qiagen AG, Switzerland) was 4–6 ng of DNA (4.5 and 3 μl per focal snail and hatchling, respectively) in a total volume of 10 μl following the protocol of Qiagen (2003). PCR-mixtures were preheated at 95°C for 15 min, followed by 30–35 cycles of 95°C for 30 s, locus specific annealing for 30 s and 72°C for 30 s (Armbruster *et al.*, 2005). PCR was finished with an extension of 8 min at 72°C. Horizontal electrophoresis was performed with SEA2000™ advanced submerged-gel electrophoresis equipment. Spreadex® EL400 gels (Elchrom Scientific AG, Switzerland) were used.

Data analyses

Null alleles can significantly affect estimates of genetic relatedness of individuals and population genetic structure (Pemberton *et al.*, 1995; Dakin & Avise, 2004). We used exclusively snails with detectable allele banding. Thus, occurrence of null alleles should be relatively low and should not have influenced parentage assignment.

As a measure for mating propensity, we used the percentage of snails mating in all trials (first copulation). For statistical analyses of courtship latency, courtship duration and copulation duration we considered only records from mating pairs. We used χ^2-test to examine whether snails from the two populations differed in proportions of successful homotypic and heterotypic mating trials. We applied two-way ANOVAs with the factors population and mating partner to examine possible effects of the origin of the focal snail and the type of mating partner (homotypic *vs* heterotypic) on copulation duration and number of eggs produced (log-transformed) and hatching success of eggs (arcsin-transformed). Data on time elapsed between two copulations, courtship latency, courtship duration and the proportion of hatchlings sired by the second mating partner (P_2-value) did not fit normal distributions. Differences in these variables between homotypic and heterotypic pairs were analysed using the Kruskal–Wallis test followed by the Mann–Whitney *U*-test for group comparisons. The rate of self-fertilization was quantified using genotyped data of the offspring and their parents. All statistical analyses were conducted using SPSS® 13 (SPSS, 2006), unless otherwise noted.

RESULTS

First copulation: precopulatory mechanism

The mating groups differed in frequency of successful copulations (χ^2-test: $\chi^2 = 6.73$, df = 2, $P = 0.035$). Sixteen out of 17 (94.1%) G × G pairs and 13 of 18 (72.2%) S × S pairs copulated, but only 10 of the 18 (55.6%) S × G pairs. The heterotypic S × G pairs showed a lower frequency of successful matings than the homotypic pairs (both populations combined; $\chi^2 = 4.56$, df = 1, $P = 0.032$), while the two groups of homotypic pairs did not differ in frequency of successful matings ($\chi^2 = 2.95$, df = 1, $P = 0.09$). This suggests the occurrence of a precopulatory isolation mechanism between snails from the two distant populations. Snails from the two populations showed slight but nonsignificant differences in mating propensity (population G: 80.8%, population S: 66.7%; $\chi^2 = 2.94$, df = 1, $P = 0.10$).

The three groups of mating pairs differed in copulation duration (Table 2; ANOVA; $F_{2,36} = 5.23$, $P = 0.010$). Copulation lasted longer in G × G pairs than in both S × S and G × S pairs (Tukey's test: $P = 0.034$ and $P = 0.021$). Copulation duration was similar in S × S and G × S pairs ($P = 0.93$). The three groups of mating pairs did not differ in courtship latency (Table 2; Kruskal–Wallis test: $\chi^2 = 3.32$, df = 2, $P = 0.19$) nor in courtship duration ($\chi^2 = 3.73$, df = 2, $P = 0.16$).

Breaks in mating behaviour were observed in 25 of the 39 (64.1%) copulating pairs (Table 2). The two homotypic groups did not differ in the percentage of pairs showing mating breaks (χ^2-test: $\chi^2 = 0.77$, df = 1, $P = 0.38$). However, a higher percentage of heterotypic S × G pairs had breaks during mating than homotypic pairs (Table 2; all homotypic pairs combined: $\chi^2 = 3.92$, df = 1, $P = 0.048$). Considering exclusively pairs with mating breaks, the different mating groups did not differ in number of breaks (Kruskal–Wallis test: $\chi^2 = 0.14$, df = 2, $P = 0.93$).

First copulation: postcopulatory mechanism

In the period between first and second copulation (mean = 28.7 days, range: 7–71 days), 20 of the 78 (25.6%) focal snails deposited eggs. In both populations, the proportion of snails that produced eggs between the two copulations was not influenced by the type of mating partner (homotypic *vs* heterotypic; χ^2-test, population G: $\chi^2 = 0.74$, df = 1, $P = 0.39$; population S: $\chi^2 = 0.003$, df = 1, $P = 0.96$). Furthermore, the number of eggs produced was not influenced by the origin of the focal snail and the type of mating partner (grand mean ± SD: 39.4 ± 21.0; two-way ANOVA; population: $F_{1,16} = 0.39$, $P = 0.54$; mating partner: $F_{1,16} = 0.09$, $P = 0.77$). The time elapsed between copulation and first oviposition averaged 16.1 days (range: 7–29 days). The number of eggs produced decreased with time elapsed between copulation and first oviposition (Spearman's rank correlation: $r_S = -0.65$, $n = 20$, $P = 0.002$). Two of the 20 individuals (both partners of S × S copulation) produced infertile eggs. In the remaining 18 snails, hatching success of eggs averaged 67.7% (range: 19.2–100.0%). Hatching success was affected neither by the origin of the focal snail nor by the type of mating partner (two-way ANOVA; population: $F_{1,14} = 0.08$, $P = 0.78$; mating partner: $F_{1,14} = 0.01$, $P = 0.93$). This suggests that individuals from the distant populations have maintained reproductive compatibility.

To examine whether self-fertilization occurs in offspring produced after the first mating, paternity was determined in a subsample of four families (a total of 116 hatchlings). In two of the four mothers examined a low frequency of selfing was found (2.6% of all hatchlings): 1 hatchling out of 33 siblings (3.0%) of one mother from population S and 2 out of 23 siblings (8.7%) from one mother from population G.

Second copulation: precopulatory mechanism

In all, 37 of the 106 (34.9%) potential mating pairs copulated. In both populations, homotypic and heterotypic matings occurred in similar frequencies (population G: $\chi^2 = 1.46$, df = 1, $P = 0.23$; population S: $\chi^2 = 0.04$, df = 1, $P = 0.83$). However, the sequence of mating partners affected the proportion of successful second copulations in population G. Focal snails that copulated first with a homotypic partner remated more frequently with a homotypic partner than snails that mated first with a heterotypic partner (71.4% *vs* 27.8%; $\chi^2 = 6.03$, df = 1, $P = 0.014$). Considering exclusively focal snails from population G that first mated with a homotypic partner, remating with a homotypic partner occurred more frequently (71.4%) than with a heterotypic partner (30.0%; $\chi^2 = 5.67$,

Table 2. Duration of courtship latency, courtship and copulation in homotypic and heterotypic matings of *Arianta arbustorum* from two distant populations (G and S).

Mating pairs	*m*	*n*	Duration (in min) of			Breaks in mating behaviour	
			Courtship latency	Courtship	Copulation	Occurrence in *n* pairs (%)	Mean number of breaks (distribution over stages in %)
First mating							
G × G	17	16	141 ± 107 (0–440)	146 ± 102 (60–500)	215 ± 84 (60–320)	10 (62.5)	1.2 (8.3; 25.0; 66.7)
S × S	18	13	189 ± 109 (0–320)	158 ± 54 (40–240)	142 ± 59 (60–260)	6 (46.2)	1.2 (42.9; 0; 57.1)
G × S	18	10	200 ± 155 (40–560)	166 ± 62 (40–240)	134 ± 63 (60–220)	9 (90.0)	1.4 (46.1; 23.1; 30.8)
Second mating							
G(×G) × S	14	6	173 ± 100 (40–320)	160 ± 80 (80–280)	210 ± 84 (60–300)	3 (50.0)	2.0 (50.0; 0; 50.0)
G(×S) × G	18	5	132 ± 59 (40–200)	180 ± 69 (80–240)	256 ± 116 (100–380)	3 (60.0)	1.0 (33.3; 33.3; 33.3)
G(×G) × G	14	10	208 ± 107 (80–420)	154 ± 75 (80–320)	194 ± 83 (100–300)	3 (30.0)	1.0 (33.3; 0; 66.7)
S(×S) × G	20	5	268 ± 138 (140–460)	208 ± 133 (100–440)	140 ± 113 (60–300)	3 (60.0)	1.3 (50.0; 25.0; 25.0)
S(×G) × S	18	5	220 ± 94 (100–300)	148 ± 58 (80–220)	144 ± 95 (60–280)	3 (60.0)	1.7 (60.0; 20.0; 20.0)
S(×S) × S	18	6	187 ± 140 (0–400)	130 ± 86 (40–280)	163 ± 75 (60–260)	0 (0)	–

Snails were allowed to mate twice. Mean values ± SD are given with ranges in parentheses; *m* indicates the number of mating trials and *n* the number of successful copulations. Data from both matings are shown separately. The occurrence of breaks in mating behaviour and the mean number of breaks per snail are also presented.

Table 3. Patterns of paternity in offspring of nine focal snails of *Arianta arbustorum* that mated twice.

Focal snail: population, mother	Mating sequence		Number of hatchlings genotyped	Number of selfed hatchlings	P_2-value	Paternity distribution		Rate of self-fertilization	Hatching success of eggs (%)	Time elapsed between copulations (days)
	First	Second				χ^2	P			
G1	G	S	14	0	0	14.00	**<0.001**	0	42.4	30
G2	S	G	6	5	0	–	–	0.83	18.2	6
G3	S	G	34	0	0.94	26.47	**<0.001**	0	87.2	6
G4	S	G	16	0	0	16.00	**<0.001**	0	47.1	22
G5	G	G	36	0	0.66	4.00	**0.046**	0	80.0	7
G6	G	G	17	2	0.33	1.67	0.20	0.12	77.3	33
G7	G	G	28	0	0.64	2.29	0.13	0	84.8	36
G8	G	G	44	0	0.50	<0.001	1.00	0	91.7	21
S1	G	S	19	1	0.44	0.22	0.64	0.05	80.0	13

Focal snails from two populations (G, Gantrisch; S, Strela) copulated with homotypic or heterotypic partners in different order. The proportion of offspring sired by the second mate is indicated by P_2. χ^2-tests were used to examine the null hypothesis that both partners contributed equally to the fertilization of eggs ($P_1 = P_2 = 0.5$; in each family df = 1), significant P-values are in bold. The rate of self-fertilization, hatching success of eggs and time elapsed between the two copulations are also presented. Hatchlings from self-fertilization were excluded from the calculation of P_2-values.

df = 1, $P = 0.017$). Similar effects of mating partner sequence were not found in focal snails from population S (in all comparisons, $P > 0.7$).

The time elapsed between first and second copulation ($\bar{x}$ = 28.7 days) differed neither between pairs of the two populations nor between homotypic and heterotypic pairs in the second mating ($n = 37$, Mann–Whitney U-test; origin of focal snails: $z = 0.15$, $P = 0.88$; type of mating: $z = 1.63$, $P = 0.10$). However, the time elapsed between the first and second copulation was influenced by the type of mating partner in the first copulation: Focal snails from population G remated sooner when the first mate was a heterotypic partner than when the first mate was a homotypic partner (13.6 *vs* 32.5 days, $n = 21$, Mann–Whitney U-test, $z = 2.40$, $P = 0.016$).

Independent of type of mating partner, focal snails did not differ in copulation duration, courtship latency and courtship duration (Table 2, ANOVA; copulation duration: $F_{1,35} = 0.17$, df = 1, $P = 0.68$; courtship latency: $\chi^2 = 1.77$, df = 3, $P = 0.62$; courtship duration: $\chi^2 = 1.68$, df = 3, $P = 0.64$).

In the second copulation, breaks in mating behaviour were observed in 15 of the 37 (40.5%) focal snails (Table 2). In both populations, homotypic and heterotypic copulations did not differ in number of breaks (Table 2; $\chi^2 = 1.81$, df = 3, $P = 0.58$).

Second copulation: postcopulatory mechanism

Only 11 out of the 37 focal snails that mated twice laid eggs after the second copulation [8 of 21 (38.1%) focal snails from population G and 3 of 16 (18.8%) focal snails from population S]. The sample sizes allowed only statistical comparisons in focal snails with different mating history from population G.

Focal snails from population G deposited on average 35.9 eggs (range: 22–48 eggs, $n = 8$). The number of eggs produced was not influenced by the mating history of the focal snail ($F_{2,5} = 0.03$, $P = 0.97$). The hatching success of eggs, however, tended to be lower in focal snails with one heterotypic and one homotypic partner than in focal snails with two homotypic partners (48.7% *vs* 84.0%; $t = 2.35$, $n = 8$, $P = 0.057$).

Paternity in double-mated snails

Paternity was analysed in a total of 214 offspring from nine focal snails that mated twice (Table 3). The proportions of offspring included in the paternity analysis averaged 99.1% (between-family range 95.0–100.0%) of the total number of hatchlings produced.

The proportion of hatchlings sired by the second mating partner (P_2-value) averaged 0.39 with a range of 0–1.00 ($n = 9$). Considering exclusively snails from population G, P_2 was 0.38 (range 0–1.00; $n = 8$). P_2 was not influenced by the mating order of heterotypic and homotypic partners (Kruskal–Wallis test, $\chi^2 = 2.17$, df = 2, $P = 0.34$). Highly skewed paternity patterns were found in the progeny of four out of the nine (44.4%) focal snails (Table 3). P_2 was not correlated with the time elapsed between the two copulations (Spearman's rank correlation, $r_S = -0.24$, $n = 9$, $P = 0.54$). However, P_2 increased with increasing hatching success of the eggs ($r_S = 0.84$, $n = 9$, $P = 0.003$).

A low frequency of self-fertilization was found in the offspring (3.7% of all hatchlings) of three out of the nine focal snails (Table 3). Two of the three mothers with self-fertilization had a heterotypic snail as a first mating partner, while the remaining mother copulated with two homotypic partners. Snails with partial self-fertilization produced fewer eggs than those with exclusive cross-fertilization (Mann–Whitney U-test, $z = 2.10$, $n = 9$, $P = 0.036$).

DISCUSSION

We investigated precopulatory and postcopulatory isolation mechanisms between individuals of the simultaneously hermaphroditic land snail *Arianta arbustorum* from two geographically distant populations. Overall, we found evidence for partial precopulatory isolation between the populations. However, paternity analyses in offspring of double-mated snails revealed that reproductive compatibility is still maintained.

In the first mating, a significant lower frequency of successful copulations was recorded in heterotypic than in homotypic pairs. A lower copulation frequency in heterotypic matings could be explained by differences in population-specific mating propensities (Fearnley, 1996). In our study, however, mating propensity did not differ between the two populations. This suggests that a partial precopulatory reproductive isolation has developed between individuals of the two distant populations.

Heterotypic pairs also showed more breaks during courtship than homotypic pairs. It has been suggested that during the long-lasting courtship with extensive reciprocal tactile and oral contacts the individuals are closely examining their potential mating partners (Leonard, 2006). Thus, individuals of

A. arbustorum might be able to recognize differences between homotypic and heterotypic mating partners. These differences may partly be population-specific because (within a population) individuals of *A. arbustorum* mate randomly with respect to shell size and kinship (Baur, 1992; Baur & Baur, 1997). There might be slight differences in courtship behaviour, and/or differences in the composition of components in the snails' skin or allohormones in the mucus, i.e. substances that induce direct effects without sensory identification, and pheromones. It has been demonstrated that allohormones and pheromones influence the mating process in various invertebrate species (Koene, 2005). In *Cantareus aspersus* (formerly *Helix aspersa*), components in the mucus associated with the love dart have an effect on sperm storage and thus on the fertilization of eggs (Rogers & Chase, 2001; Chase & Blanchard, 2006; Chase 2007). Mate choice experiments with individuals of the closely related land snails *Bradybaena pellucida* and *B. similaris* revealed that reproductive isolation was associated with differences in sexual pheromones (Wiwegweaw *et al*., 2009a).

In the second mating trial, homotypic and heterotypic pairs did not differ in frequency of successful copulations or in courtship behaviour. Three different factors may explain these findings. In the first mating, virgin snails were allowed to copulate, while in the second mating the focal snails were experienced and stored sperm from the previous copulation. This might have influenced their mating behaviour (Kokko & Mappes, 2005). In *A. arbustorum* one copulation is sufficient to fertilize all the eggs produced by one individual in a reproductive season (Chen & Baur, 1993). Further matings increase the genetic variability in the offspring but are not mandatory for the fertilization of all eggs. Thus, the first mating partner should be more carefully chosen than the following partners. Alternatively, virgins might be less choosy about the first mating partner in order to begin as soon as possible with egg production in the time-limited reproductive season (Arnqvist & Rowe, 2005).

Another explanation relates to quantity and/or quality traits of the sperm stored from the first partner. The quality and/or quantity of sperm received could influence the mating propensity and courtship behaviour in the second mating. Sperm quantity might be of minor importance because only a small fraction (0.02–0.1%) will actually be stored in the spermatheca (Lind, 1973; Roger & Chase, 2001). In our study, we found that snails which mated first with a heterotypic partner copulated sooner with a second partner than snails with a homotypic partner in the first copulation. This suggests that snails recognized their mating partner and assigned a heterotypic partner a lower quality than a homotypic partner. Furthermore, our finding could be explained as a consequence that homotypic partners may more effectively inhibit their partners from engaging in further matings than heterotypic partners to increase the fertilization success of their own sperm (Koene, Brouwer & Hoffer, 2009; Koene *et al*., 2010).

The present study showed that snails that copulated first with a homotypic partner remated more frequently with a homotypic partner than snails which copulated first with a heterotypic partner in one of the two populations (the same effect was not found in population S with a smaller sample size). This indicates that previous mating encounters may influence prospective mate choice in *A. arbustorum*. Such mechanisms have already been observed in *Drosophila paulistorum*, where earlier mating experience with partners from the same population promoted a preference for homotypic mates (O'Hara, Pruzan & Ehrman, 1976).

We could not find any influence of the origin of the mating partner on egg production. Thus, reproductive compatibility was still maintained as already reported in Baur & Baur (1992a). The study design chosen (paternity analysis) did not allow us to examine the survivorship and fitness of the emerged offspring, which could be influenced by outbreeding depression (Wiwegweaw *et al*., 2009b). However, we recorded a reduced hatching success in eggs of focal snails which copulated with one heterotypic and one homotypic partner compared to eggs of focal snails which mated twice with homotypic partners. This may indicate a slight postcopulatory isolation between individuals of these geographically distant populations.

Sperm precedence and sperm utilization

In the present study, paternity analyses of the progeny of double-mated snails revealed no influence of the origin of the mating partner. Thus, the observed partial precopulatory reproductive isolation did not translate into sperm precedence and sperm utilization. The proportion of hatchlings sired by the second mate (P_2) averaged 0.39 indicating a first-mate advantage. In a previous study, using shell colours as genetic markers, P_2 of double-mated individuals of *A. arbustorum* averaged 0.34 (Baur, 1994a). Nonrandom distributions and highly skewed paternity patterns in the progeny (44.4% of the double-mated snails) could be a result of precopulatory and/or postcopulatory sexual selection, e.g. sperm competition or cryptic female choice (Birkhead & Møller, 1998). In helicid snails, precopulatory selection processes could be due to premating behaviour like dart shooting, i.e. piercing the mating partner with a mucus-coated calcareous dart which enhances the sperm storage of the recipient (Koene & Schulenburg, 2005; Chase, 2007). However, dart shooting is not an obligatory courtship element of *A. arbustorum*. In laboratory tests, only 50% of the copulating individuals used or tried to use the dart (Bojat & Haase, 2002). Postcopulatory processes could be possible in *A. arbustorum* considering the morphology and complex muscular network of the sperm storage organ (spermatheca; Baur, 2007). This allows differential sperm storage and utilization (Bojat, Dürrenberger & Haase, 2001; Bojat, Sauder & Haase, 2001). Sperm from different fathers could be stored separately in different tubules of the spermatheca and consequently, in a further step, might be used separately for the fertilization of eggs (Haase & Baur, 1995; Bojat & Haase, 2002).

The skewed paternity pattern and first-mate sperm precedence recorded in the present study might be a result of sperm competition, selective storage and/or use of sperm by the focal snail. Mating order seems to be more important for the paternity pattern than the origin of the mating partner.

Self-fertilization

In simultaneous hermaphroditic pulmonate gastropods, sperm and eggs of one individual are present at the same time but stored in different storage localities (Baur, 1998). However, under certain circumstances self-fertilization may occur. In the present study, genetic analyses revealed a low frequency of self-fertilization (3.7% of all hatchlings) in the progeny of double-mated individuals of *A. arbustorum*. In a laboratory experiment, individuals of *A. arbustorum* isolated for 2–3 years produced a few self-fertilized eggs (1–2% of the eggs of the mated snails) with low hatching success (Chen, 1994). A low frequency of self-fertilization was also recorded in one of four natural populations of *A. arbustorum* (Kupfernagel *et al*., 2010). However, in *A. arbustorum* outcrossing is preferred to self-fertilization even under outbreeding circumstances, as observed in this study. This result indicates that potential negative fitness consequences due to outbreeding depression are less costly than inbreeding depression as a result of self-fertilization (Frankham, 1995; Wiwegweaw *et al*., 2009b).

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Chapter 5

An improved immunocytochemical labelling technique to study the fate of sperm in the reproductive organs of gastropods

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Submitted, in revision

An improved immunocytochemical labelling technique to study the fate of sperm in the female reproductive organs of gastropods

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Abstract

The mechanisms of sperm transfer, sperm storage and sperm utilization and digestion are crucial for the understanding of processes of postcopulatory sexual selection. Previous studies analysing paternity in offspring have generally been focusing only on the ultimate outcome of the interactions between male and female sexual selection. For a mechanistic understanding of the fate of received sperm and the involved patterns of postcopulatory sexual selection new techniques are required. Here, we present an improved immunocytochemical sperm-labelling technique to track the fate of 5-bromo-2'-deoxyuridine (BrdU)-labelled sperm in the female reproductive organs of invertebrate species. The technique presented was tested in individuals of the simultaneously hermaphroditic land snail *Arianta arbustorum* L., 1758. We determined the percentage of labelled sperm in spermatophores delivered and assessed the constancy of detecting labelled sperm in the reproductive organs of the receivers: spermatheca (sperm storage organ) and bursa copulatrix (sperm digestion organ). In our tests, the proportion of sperm labelled among the sperm produced by an individual averaged 99.3%. Furthermore, labelled sperm could be consistently visualized in both the sperm storage and the sperm digestion organ of all receivers examined.

The sperm-labelling technique can be combined with traditional sperm staining techniques, which allows to distinguish between sperm from two males in the reproductive tract of a double-mated female. It can easily be adjusted to other invertebrate species providing insight into mechanisms of sperm competition and cryptic female choice. The technique may contribute to our understanding of the evolution of male and female reproductive morphology and physiology in a variety of species.

Key-words: *Arianta arbustorum*, female reproductive organs, Gastropoda, immunostaining, postcopulatory sexual selection, spermatheca, sperm digesting organ, sperm storage

Introduction

Sexual selection continues after copulation in form of sperm competition and female-sperm manipulation. Sperm competition occurs when spermatozoa from different males compete in the reproductive tract of a female for the fertilization of her eggs (Parker 1970). In a variety of species, females have a physiologically and morphologically complex reproductive system, which may enable them to control or influence offspring paternity by postcopulatory sperm storage and selective sperm use (frequently summarized as cryptic female choice; Eberhard 1996, Birkhead and Møller 1998). An important challenge of current research is to identify the relative impact of different components of postcopulatory sexual selection (e.g. Eberhard 2004; Chapman et al. 2003; Arnqvist and Rowe 2005). The different processes are often difficult to partition because they operate simultaneously and may produce similar outcomes.

A key issue in studying mechanisms of sperm competition and cryptic female choice is the understanding of the patterns of sperm storage, sperm use and sperm digestion (Birkhead and Møller 1998). Current studies infer these patterns mainly from paternity data, which only reveal the ultimate outcomes of the interactions between male and female reproductive processes (Schärer et al. 2007). With a mechanistic understanding of the fate of received sperm, and the involved patterns of postcopulatory sexual selection, we can better understand the evolution of male and female reproductive morphology and physiology.

Different approaches have been applied to track the donor's sperm in the recipient. Using radiolabelled nucleotides, which result in a specific labelling of the DNA of the donor's sperm, the position of sperm in the female tract can be located by scintillation counting or autoradiography (*e.g*. Beeman 1970; Nollen 1975; Bishop 1996). However, this approach has several limitations. Recently, insertions of fluorescence genes in the genome of *Drosophila melanogaster* Meigen, 1830, have been used to distinguish sperm from different mates (Manier et al. 2010). However, the insertion of genes requests an extensive knowledge of the genome of the target species that is currently only available for few species. In recent years, immocytochemical techniques have been developed to visualize particular types of cells. For example, 5-bromo-2'-deoxyuridine (hereafter called BrdU), a synthetic analogue of the nucleic acid component thymidine is incorparted into newly synthesized nucleic acids substituting thymidine and can be visualized with immunocytochemical staining methods. This technique is commonly

used for cancer cell detection in humans and in cell development research (Soames et al. 1994). In a few gastropod species, BrdU has been used to investigate brain cell development and the immune defense system (Zakharov et al. 1998; Gorbushin and Iankovleva 2006). Schärer et al. (2007) used the BrdU-immunocytochemical approach to track sperm in the tiny transparent flatworm *Macrostomum lignano*. Individuals of this free-living flatworm were labelled by incubation in BrdU-enriched artificial seawater. BrdU-uptake occurred through the skin. There was a considerable mortality of flatworms in the staining process (36%) and no data on the percentage of sperm labelled have been reported (Schärer et al. 2007). We further developed the BrdU-labelling method to track sperm received in the female organs of more complex invertebrates. As model organism we used the simultaneous hermaphrodite land snail *Arianta arbustorum* L., 1758.

In stylommatophoran gastropods, the site of storage (= spermatheca) of received sperm (allosperm) is of particular interest. It shows an enormous variability in structure, morphology and number of tubules (reviewed in Baur 2010). For example, *Oxychilus draparnaudi* Beck (1837) and *Bradybaena fruticum* Müller (1774) have a single spermathecal tubule. In *Succinea putris* L. (1758) two spermathecal tubules occur, and 34 tubules have been recorded in the spermatheca of *Drymaeus papyraceus* Mawe (1823). There is also a considerable within-species variation in the number of spermathecal tubules (e.g., 3–5 in *Helix pomatia* L., 1758; 4–19 in *Cornu aspersum* Müller, 1774; Evanno and Madec 2007; Koemtzopoulos and Staikou 2007). As a consequence of the large intraspecific variation in the number of spermathecal tubules, different individuals might have different possibilities to store allosperm from more than one mating partner. Mixing of sperm from different mates would be more likely in a less structured spermatheca, whereas a large number of tubules could potentially allow better separation of spermatozoa from different mates.

In stylommatophoran gastropods, the sperm received travel through the spermoviduct to the spermatheca. The majority of sperm, however, are directed to the sperm-digesting bursa copulatrix. As a consequence, only a minor fraction of the received allosperm may reach the storage organ (Lind 1973; Rogers and Chase 2001). In the spermatheca, allosperm are stored for long periods (up to 4 years; Baur 1998). Sperm survive best when attached to the walls of spermathecal tubuli (Chase and Darbyson 2008).

We further developed the BrdU-labelling method by injecting BrdU to growing individuals of *A. arbustorum* and by conducting several tests to assess the constancy of both sperm labelling in the donor and the detection of labelled sperm in different organs of the sperm-receiving mating partner.

Material and Methods

MODEL ORGANISM

Arianta arbustorum is common in moist habitats of northwestern and central Europe (Kerney and Cameron 1979). The snail has determined growth (shell width of adults 17–22 mm). Individuals become sexually mature at 2–4 years and adults live another 3–4 years (Baur and Raboud 1988). Mating is random with respect to shell size, different degrees of relatedness and mating history (Baur 1992; Baur and Baur 1997; E. Häussler, unpubl. data). Copulation is reciprocal; both snails transfer simultaneously one spermatophore. The number of sperm delivered varies considerably (803,000–3,969,000) with an average of 2,185,000 (Baur et al. 1998). However, studies in other helicid snails revealed that only a minor fraction of sperm received are stored in the spermatheca (0.02–0.10%; Lind 1973; Rogers and Chase 2002). The majority of sperm is digested in the bursa copulatrix.

The sperm storage organ of *A. arbustorum* shows a considerable variation in the number of blind-ending spermathecal tubules (2–9) uniting to a common duct, which opens into the fertilization chamber (Baminger et al. 2000; Beese et al. 2009). Incoming sperm are not equally distributed among all spermathecal tubules suggesting that sperm from different partners might be stored separately (Baur 1994*a*; Haase and Baur 1995; Bojat and Haase 2002). The musculature surrounding the spermathecal tubules is arranged in a complex three-dimensional network (Bojat et al. 2001). If there were a selective activation of the muscles of each tubule (which has not yet been examined), this might allow the animal to utilize selectively sperm stored in single tubules and thus promotes a selective fertilization of eggs (Baur 2010).

In *A. arbustorum*, the dominant mode of reproduction is cross-fertilization. Though, in the absence of a potential mating partner, a low rate of self-fertilization can be observed both in the laboratory and in natural populations (Chen 1994; Kupfernagel et al. 2010). *Arianta arbustorum* mates repeatedly in the course of a reproductive season and fertile sperm from different partners can be stored for more than 1 year in the sperm

storage organ (Baur 1988). There is a probability of 5–8% that a copulation will not lead to fertilization of eggs (no sperm transfer or transfer of infertile sperm; Chen and Baur 1993).

SNAIL SAMPLING AND MAINTENANCE

Virgin (subadult snails with a shell diameter of 8–11 mm) and adult individuals of *Arianta arbustorum* (shell diameter: 15–19 mm) were collected in the subalpine forest of Gurnigel, Switzerland (46° 45.5' N, 7° 27.5' E, elevation 1230 m a.s.l.) on 14 May 2009, approximately 4 weeks after hibernation. Adult individuals might therefore have stored allosperm from matings in the ongoing and previous year(s).

Snails were kept individually in transparent beakers (8 cm deep, 6.5 cm in diameter) lined with moist soil (approximately 4 cm) at 19 °C with a light:dark cycle of 16:8 h. Fresh lettuce was provided twice a week and at the same time the beakers were cleaned. Subadult snails were raised singly until they reached sexual maturity as indicated by the formation of reflected lip at the shell aperture. The snails were marked individually by writing numbers on their shells with a waterproof felt-tipped pen on a spot of correction fluid (Tipp-Ex). The animals showed no visible reaction to the marking procedure.

SPERM LABELLING

Subadult snails were randomly assigned either to a treatment group (hereafter called BrdU-labelled) or to a control group (hereafter called unlabelled). Individuals of the BrdU-labelled group received a BrdU injection (5-bromo-2'-deoxyuridine, ROTH AG, Arlesheim. Switzerland; 1% in Ringer solution) every second week until they reached sexual maturity. Using a syringe, 150 μl solution was injected into the foot muscle, next to the refractory muscle and shell chamber. Absorption of BrdU due to skin immersion and/or injection has already been described in other invertebrate species (Zakharov et al. 1998; Gorbushin and Iakovleva 2006; Schärer et al. 2007). BrdU is mainly incorporated into newly synthesized nucleic acid. Consequently, repeated BrdU-treatments until the individuals attained sexual maturity ensured that all the continuously produced sperm are labelled. A further BrdU injection was given 24–36 hours after successful copulation to label replenishing sperm.

To examine any potential influence of BrdU on snail size, the shell width of BrdU-labelled and unlabelled individuals was measured after copulation to the nearest 0.1 mm using vernier callipers.

IMMUNOSTAINING

Immunostaining was done with sperm delivered in the spermatophore by BrdU-labelled snails and with labelled sperm distributed in the female reproductive organs of unlabelled receiver individuals. Labelled sperm were extracted from the spermatophore of a donor snail and mounted on HistoBond® microscope slides for air-drying and trypsin treatment (see below). To examine the fate of sperm in the female organs of the receiver, focal unlabelled individuals were allowed to mate with BrdU-labelled individuals (details see below). After successful copulation, focal snails were killed by deep freezing (-80 °C) and their female reproductive organs were dissected and fixed for 2–4 h in paraformaldehyde (4%). Then the reproductive organs were rinsed in distilled water (30–60 min), incubated in 50% ethanol (15 min) and finally stored in 70% ethanol. For immunostaining and structural analyses, the dissected organs were embedded in paraplast and serially sectioned at 8 μm and positioned on HistoBond® microscope slides. The slides were then deparaffinized in xylene and rehydrated through graded ethanol solutions to phosphate-buffered saline (PBS).

Mounted tissue slices were treated with 0.1% trypsin (in EDTA; INVITROGEN AG, Basel, Switzerland) for 20 min at 37 °C, washed 5 min in PBS-T (PBS with 1% Triton X-100, ROTH AG, Arlesheim, Switzerland), followed by a treatment with 2 *N* HCl for 30 min at room temperature to denature the DNA. After rinsing in PBS-T, enzyme activity and DNA denaturation were blocked with BSA-T (= PBS-T with 1% albumin fraction V, ROTH AG, Arlesheim, Switzerland) for 15–30 min at 37 °C. Nucleic acid incorporated BrdU was stained with an in-mouse-produced primary monoclonal antibody directed against single-stranded DNA containing brome-associated-uridine (BU-33, SIGMA, Missouri, USA; diluted 1:50 in BSA-T) incubated over night at room temperature (alternatively 4–5 h at 37 °C), washed four times in PBS-T, followed by a 15 min unspecific binding reducing treatment with goat serum (G9023, SIGMA, Missouri, USA) at 37 °C, and 1 h incubation at 37 °C with a goat-anti-mouse FITC-conjugated secondary antibody (1:100, F5897, SIGMA, Missouri, USA) directed specifically against the tissue bounded BU-33 antibodies. With the

incubation of the second antibody the cell nuclei marker DAPI (1:500, ROTH AG, Arlesheim, Switzerland) was added for counterstaining. Generally, DAPI is embedded in the DNA of cells and allows the determination of unlabelled sperm in the focal snail, because its incorporation is inhibited in BrdU-labelled cells.

After washing steps (three times with PBS-T), tissue slides were embedded in PBS-glycerol (ratio 1:1) with the anti-microbial substance Thimerosal (0.005%, ROTH AG, Arlesheim, Switzerland) and coverslipped. After staining, the microscope slides were stored at 4 °C for up to 7 days at darkness to prevent fading of fluorescence.

For fluorescence visualization of BrdU-labelled sperm and cells, a microscope (Olympus AX-70) with magnifications ranging from 40 to 400 was used. For digital photography the imaging software SPOT version 4.6 (Diagnostic Instruments Inc., Michigan 2006) was applied. For each sperm donor, the percentage of labelled sperm was calculated using photographs of four randomly chosen sections containing 29–488 immunostained sperm per section (Table 1) of the microscope slides. For sperm counting on the digital photographs, the open source programme ImageJ version 1.44 (http://rsbweb.nih.gov/ij/) was applied. This software facilitates manual counting and particle analysis from photographs. The repeatability of the sperm counting technique was assessed by using multiple measurements ($N = 4$) in randomly-chosen sections ($N = 3$) on photographs following Lessells and Boag (1987).

A series of tests were conducted to assess the constancy of the labelling method.

TEST 1: PROPORTION OF SPERM LABELLED

Two sets of mating trials were conducted to assess the percentage of labelled sperm among all sperm produced by individuals treated with BrdU. Virgin unlabelled individuals (focal snails, V) were paired with virgin BrdU-labelled (V_L) individuals (V x V_L). In three randomly chosen focal snails, the spermatophore received from the BrdU-labelled partner was dissected after copulation (see below). To examine the percentage of labelled sperm in sperm newly produced after a copulation, the BrdU-labelled snails were allowed to replenish their autosperm reserves for 7–10 days after mating with an additional BrdU-injection 1–2 days after the first copulation. These snails (now non-virgin but labelled, NV_L) were again paired with virgin unlabelled individuals (V x NV_L) and the spermatophores of the BrdU-labelled snails were dissected out of the focal snails. Furthermore, we paired unlabelled individuals with

unlabelled focal individuals (N = 6) to obtain spermatophores for a comparison of the total number of sperm delivered (labelled vs. unlabelled).

Sperm were extracted from the obtained spermatophores as described in Locher and Baur (1997). The sperm mass delivered by each BrdU-labelled individual was divided into two equal aliquots. The first was used for estimating the total number of sperm delivered with the spermatophore following the procedure of Locher and Baur (1997). The second aliquot was used for immunostaining. Sperm were stored at –20 °C until preparation for staining. From the sperm solution, 2 μl were loaded on HistoBond® microscope slides (Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany) and air-dried for immunostaining. After immunostaining (Figure 1), we counted the number of labelled (BrdU with conjugated fluorescence marker FITC) and unlabelled sperm (DNA with fluorescence marker DAPI) on four randomly chosen slide sections for each spermatophore (see above, Figure 1).

TEST 2: BRDU-LABELLED SPERM IN THE FEMALE ORGANS OF THE RECEIVER

Mating trials were conducted to check the constancy of immunostaining of labelled sperm in the female organs of unlabelled sperm receivers. The spermatheca and bursa copulatrix of six previously virgin, unlabelled individuals mated with a BrdU-labelled snail were dissected 36–48 h after copulation. This period of time allows sperm to leave the spermatophore and to reach the sperm storage organ (spermatheca; cf. Lind 1973; Bojat and Haase 2002). The remaining sperm are transported into the bursa copulatrix (sperm digestion organ). In three copulating pairs the BrdU-labelled partner was a virgin snail (V_L), in the other three pairs a non-virign BrdU-labelled snail (NV_L). The focal snails were killed by freezing (-80 °C) 36–48 h after copulation. No snail had laid any eggs within 48 h after copulation.

As a control for the occurrence of auto-fluorescence, sperm, the spermatheca and bursa copulatrix of two non-labelled snails that copulated with non-labelled snails were investigated as described above.

DATA ANALYSES

All statistical analyses were conducted using PASW®19 (SPSS Inc., Chicago 2010).

Results

TEST 1: PROPORTION OF SPERM LABELLED

The spermatophore was obtained from five out of six mated BrdU-labelled snails that copulated. One BrdU-labelled snail did not deliver any spermatophore. The total number of sperm delivered in a spermatophore averaged 1.28 x 10^6 (range: 0.96 x 10^6 to 1.69 x 10^6; N = 5; Table 1) in BrdU-labelled individuals. In unlabelled control individuals the total number of sperm delivered averaged 1.13 x 10^6 (range: 0.94 x 10^6 to 1.48 x 10^6; N = 6). There was no difference in the number of sperm delivered between labelled and unlabelled individuals (t-test: t = 1.0, df = 9, P = 0.3).

The percentage of labelled sperm delivered in a spermatophore of BrdU-labelled snail averaged 99.3% (range: 98.3–99.9%, Table 1). BrdU-labelled snails that mated for the first time did not differ in the percentage of labelled sperm from those that mated for the second time (Mann-Whitney U-test: z = 1.7, N = 5, P = 0.2). The repeatability of the sperm counting method was 99.8% ($F_{2,11}$ = 1502.3, P < 0.001). These results indicate that both the labelling method presented and the timing of BrdU application are appropriate to achieve a high rate of sperm labelling.

TEST 2: BRDU-LABELLED SPERM IN THE FEMALE ORGANS OF THE RECEIVER

The spermatheca and bursa copulatrix of six unlabelled snails that copulated with a BrdU-labelled snail were checked for labelled sperm (Figs. 2 and 3). All examined individuals stored labelled sperm in the spermatheca, and all snails had partly digested labelled sperm in the bursa copulatrix. This indicates that BrdU-labelled sperm were not excluded from entering the female reproductive organs of the receiver. Furthermore, the distribution of labelled sperm found in the spermatheca and bursa copulatrix of the receiver did not differ between matings with labelled snails that copulated for the first and second time.

BRDU INFLUENCE ON SNAIL GROWTH AND SIZE

A long-term application of BrdU could negatively influence fitness traits including growth, adult size and fecundity of the animals. However, subadult snails randomly assigned either to the BrdU-labelled group or the control group did not differ in adult size attained during the experiment (labelled vs. unlabelled, mean ± SD: = 17.4 ± 0.7

mm vs. 17.7 ± 0.6 mm; t-test: t = -1.3, df = 21, P = 0.2). This indicates that the application of BrdU did not affect snail growth.

Discussion

In this study study, we further developed a sperm-labelling technique that allows tracking the fate of sperm of a single male in the reproductive organs of a female. The tests demonstrate a high efficiency of the sperm-labelling procedure and show that labelled sperm can be visualized in the female reproductive organs of the receiver with a high constancy. The immunocytochemical approach opens new ways to investigate mechanisms and processes of

postcopulatory sexual selection including sperm transfer and both selective sperm storage and sperm utilization. In particular, sperm from two different males (labelled and unlabelled) can be followed in the female reproductive tract.

In the past, studies dealing with sperm distribution in the reproductive organs of the receiver utilising traditional histological staining methods could not distinguish between sperm from different males. Moreover, sperm of radiolabelled males could not be precisely localized in the female reproductive tract (Bishop and Sommerfeldt 1996). Most recently, insertions of fluorescence genes were used to distinguish sperm of different males in the female tract in *D. melanogaster* (Manier et al. 2010). However, such transgenic transformations request a detailed knowledge of the species genome, which is so far available only for a few species. In contrast, the immunostaining technique presented may allow detecting distinctively labelled sperm in the female tract in a variety of species. The technique can be combined with traditional sperm staining methods which allows to distinguish between sperm from two males (see below). It could be continued to develop by including a second sperm labelling substance (Miller et al. 1991). Furthermore, it can be easily adjusted to other invertebrate species. In bivalves, crustaceans, several gastropod species and a fish species, BrdU has already been used to investigate cell proliferation and cell developmental processes (Moore et al. 1994; Zakharov et al. 1998; Gorbushin and Iankovleva 2006).

To successfully apply the novel immunocytochemical approach in studies of postcopulatory selection the following conditions should be considered. Any application of BrdU should be well timed because it is only incorporated in newly synthesized nucleic acid. To achieve a high rate of sperm labelling success a repeated application

during growth and developmental of testes or ovotestis is required. For a good labelling success of all cells/sperm a high saturation of BrdU in the individual is necessary.

Long-term application of BrdU increases the risk of cell changes which may lead to cell death (Caldwell et al. 2005). However, we could not detect any influence of the BrdU application on the size and mating behaviour of snails (S. Kupfernagel; personal observation). Moreover, the distribution of BrdU-labelled sperm found in the sperm storage organ of the receiver did not differ from that of unlabelled sperm stained with traditional histological methods reported in previous studies (Baminger and Haase 1999; Bojat et al. 2001*b*). This indicates that BrdU-labelling does not strongly affect physiological traits of reproduction and may therefore be well suited to study the fate of sperm in the female reproductive organs of the receiver.

We also showed that the sperm-labelling technique can be used to investigate the mechanisms of sperm digestion which might allow to follow digested particles originating from the labelled sperm in the receiver. The additional application of DAPI staining (see Material and Methods) visualizes non-BrdU-labelled sperm in the female reproductive organs of the receiver and hence allows the investigation of sperm distribution in double-mated snails, i.e. BrdU-labelled vs. unlabelled sperm in the receiver. Other DNA-labelling substances such as iododeoxyuridine (Miller et al. 1991) could be used to investigate sperm storage and sperm utilization of multiple inseminations.

To sum up, the use of BrdU for sperm labelling has many possible applications in various invertebrate species. With this technique, the fate of sperm can be followed in the female reproductive tract after copulation. In double-mated individuals, the sperm-labelling technique can give insight into mechanisms of postcopulatory sexual selection. The sperm-labelling technique presented has been developed in a hermaphroditic species. However, it could be easily adjusted to a variety of gonochoristic species. This technique may improve our understanding of the evolution of male and female morphology and physiology and postcopulatory selection in general in hermaphroditic and gonochoristic species.

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Table 1. Constancy of the BrdU-labelling method measured by the percentage of labelled sperm delivered in spermatophores of *A. arbustorum* treated with BrdU (see methods).

ID of labelled snail	Sperm from first/second copulation*	Number of labelled sperm† mean [range]	Labelled sperm (%)‡ mean [range]	Total number of sperm delivered
L1	first	84.8 [29–146]	98.3 [96.7–100.0]	1.25×10^6
L16.1	first	75.8 [45–106]	99.1 [97.9–100.0]	1.00×10^6
L11	second	300.3 [132–488]	99.4 [98.5–100.0]	1.69×10^6
L16.2	second	215.3 [146–351]	99.9 [99.4–100.0]	0.96×10^6
L19	second	224.8 [115–332]	99.7 [99.1–100.0]	1.51×10^6

* First copulation: sperm were produced during growth before the first copulation; second copulation: sperm were also produced between the first and second copulation.

† The number of labelled sperm were counted in randomly-chosen sections ($N = 3$) of photographs ($N = 4$)

‡ $N = 4$

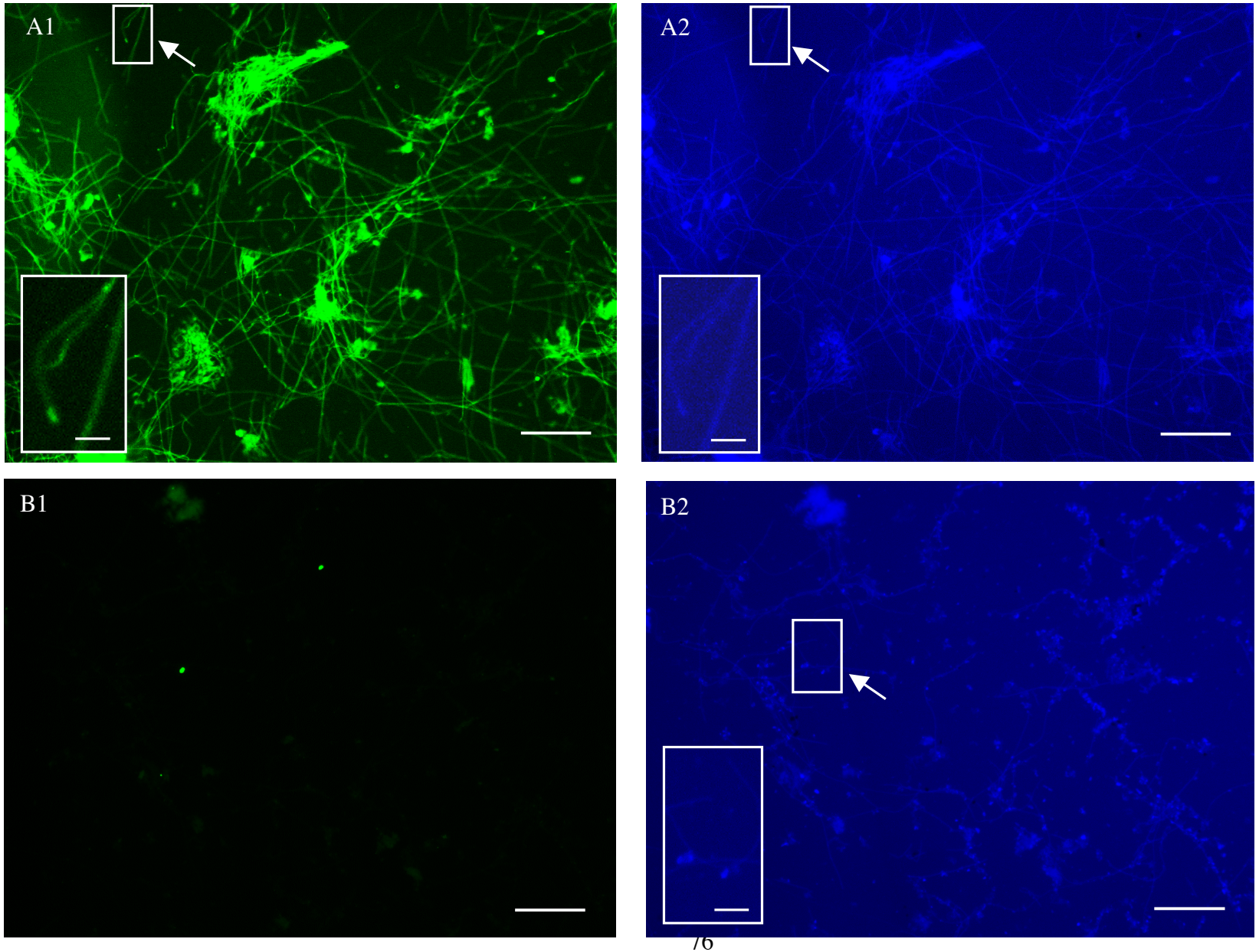
A1
A2
B1
B2

Fig. 1. Immunostained sperm of the land snail *Arianta arbustorum*. Scale bars are 200 μm, and 50 μm in the inlets. (A) BrdU-labelled sperm with fluorescence marker FITC (A1) and DNA-binding fluorescence marker DAPI (A2), (B) unlabelled sperm with BrdU/FITC (B1; negative control: no signal should be visible), and with DNA/DAPI (B2). Arrows indicate the location of a section, which is presented with higher magnifigation in the lower left corner.

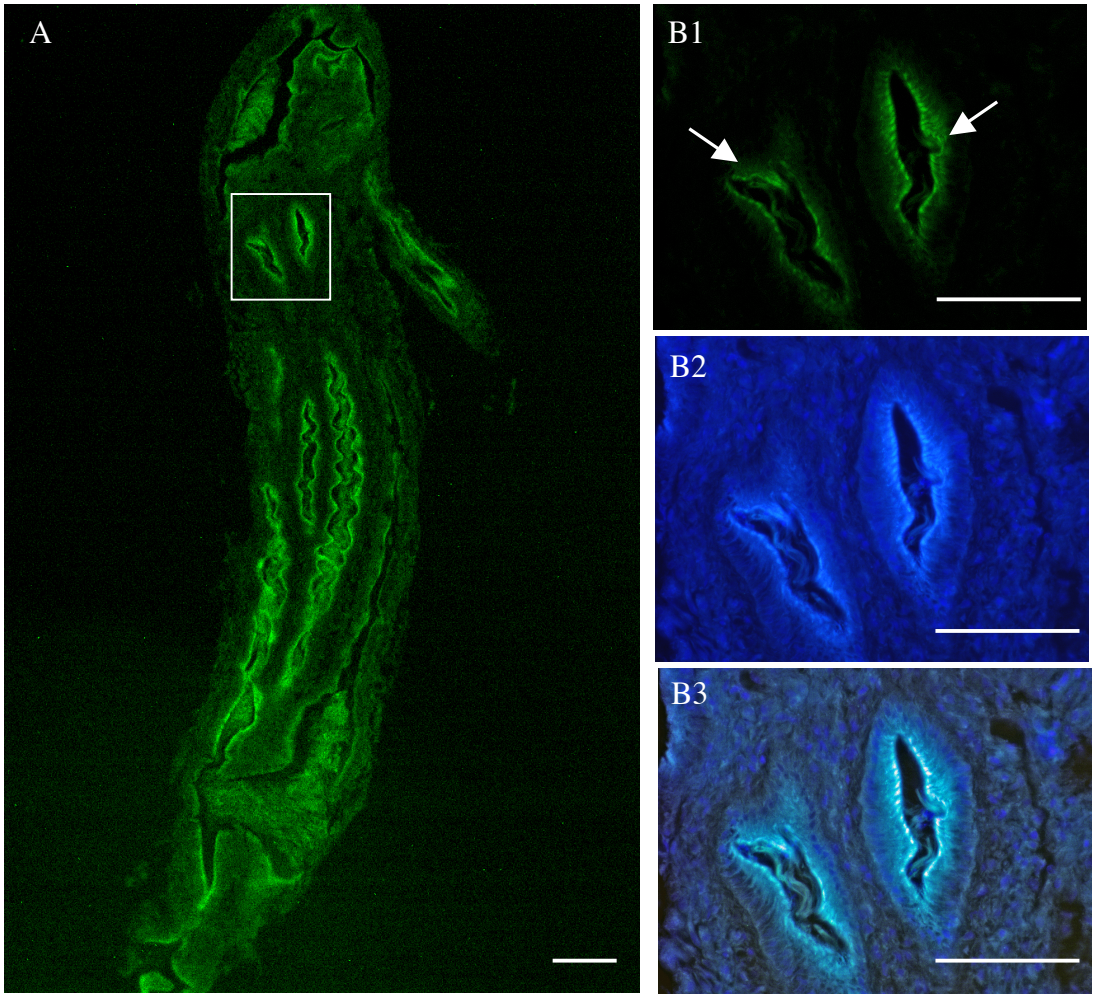

Fig. 2. Immunostained spermatheca of the land snail *Arianta arbustorum*. (A) spermatheca storing BrdU-labelled sperm, stained with FITC. (B1–3) Higher magnification view of a cross-section of tubules cross-sectioned from panel A: stained with BrdU/FITC (B1), DNA stained with DAPI (B2) and FITC & DAPI merged (B3). Arrows indicate labelled sperm associated to the wall of a spermathecal tubule. Scale bars are 200 μm.

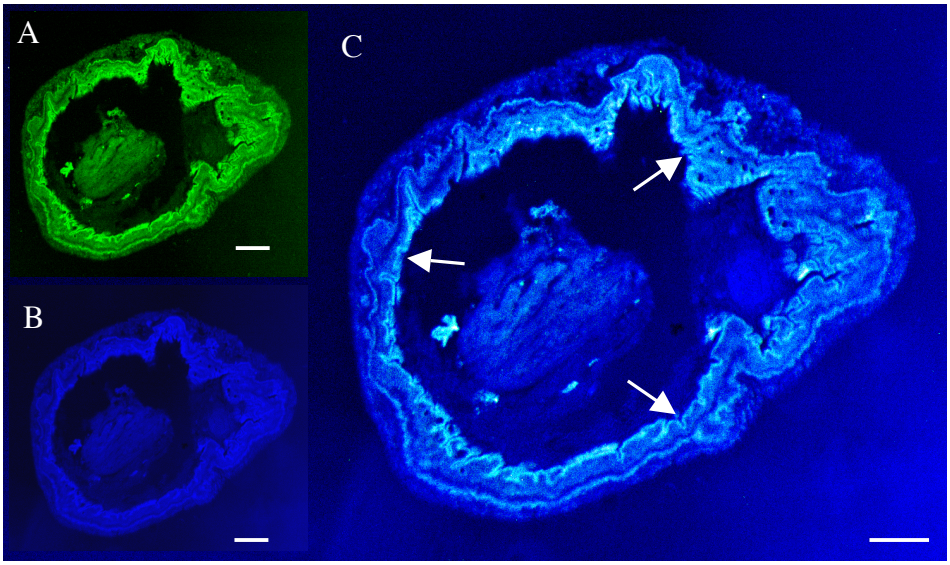

Fig. 3. Immunostained bursa copulatrix of *Arianta arbustorum* with digested BrdU-labelled sperm (remnants of these sperm are indicated by arrows). (A) BrdU with FITC, (B) DNA with DAPI, (C) FITC & DAPI merged. Scale bars are 500 μm.

GENERAL DISCUSSION

For a better understanding of evolutionary and biodiversity processes, a fundamental issue is to gain more knowledge of the impact of sexual selection. The aim of the present thesis was to improve our understanding of sexual selection mechanisms in simultaneous hermaphrodites. Particularly, we used the results of behavioural studies, findings in life history traits and genetic studies in the model organism *Arianta arbustorum* to broaden the view on mate choice and sperm utilization patterns.

In **Chapter 1**, we assessed derminants of mating success and female and male reproductive success considering mate choice and patterns of sperm utilization in individuals of *A. arbustorum*. Using video-recorded behaviour and the assignment of genotyped hatchlings to the female and male function of individual parents, we could show that mating success, which is equal to the female and male function in simultaneous hermaphrodites with reciprocal copulation, and female reproductive success (number of hatchlings emerging from the eggs laid by a focal snail) was determined by the snail's activity. Similarly, and male reproductive success (number of hatchlings sired by the focal snail) was mainly determined by the activity of an individual. Activity as measured in our study might be a proxi for the conditional state or healthiness of a snail. Individuals of *A. arbustorum* infested by parasitic mites showed a reduced activity, produced fewer eggs and exhibited a higher winter mortality than parasite-free snails (Schüpbach and Baur 2008, 2010). Consequently, a high activity and healthiness result in a large number of mate encounter, and thus influence reproduction. Furthermore, our results revealed that female reproductive success was positively correlated with male reproductive success. These results contrast basic sex allocation theory, which are based on the simplified assumption that all individuals in a population have the same fixed reproductive resource budget and thus predict a trade-off between female and male reproductive allocation (Charnov 1982). However, there is so far little empirical evidence for this trade-off in animals (Schärer 2009). In studies often no correlation (Baeza 2007) or a positive correlation (Schärer *et al*. 2005) were found between female and male allocations rather than the expected negative correlation. These findings indicate that in many cases the trade-off assumptions seem not to be justified.

Mating frequency has implications for the effective population size, rate of gene flow, genetic structure of the population and maintenance of genetic variation (Reed and Frankham 2003). **Chapter 2** provides evidence for a high level of multiple paternity in natural populations of *A. arbustorum*. Evidence for multiple paternity in helicid snails has already been obtained from controlled mating experiments (*e.g.* in *C. aspersum*, Rogers and Chase 2002, Evanno *et al.* 2005) and in wild populations using shell colour as genetic marker (Murray 1964, Baur 1994*a*). This study was, however, the first that quantitatively assessed the number of fathers involved in the progeny of single mothers of a terrestrial snail species.

The results also showed that the local density of potential mating partners affected the level of multiple paternity. However, it could not be excluded that slight among-site differences in environmental conditions influenced snail activity to a different extent resulting in different levels of multiple paternity in *A. arbustorum* (see **Chapter 1**).

The observation of the sperm utilization patterns revealed highly skewed paternity patterns in the progeny of 15 (57.7%) of the 26 mother snails which could be the result of sexual selection mechanisms occurred in the wild. Such sexual selection mechanisms could be happened precopulatory (*e.g.* mate choice and mate competition) and/or postcopulatory (*e.g.* female manipulation of incoming and stored sperm). It has been suggested that during the long-lasting courtship with extensive reciprocal tactile and oral contacts the individuals are closely assessing their potential mating partners (Leonard 2006). Although individuals of *A. arbustorum* mate randomly with respect to shell size and kinship (Baur 1992, Baur and Baur 1997), this species seems to be able to precopulatory discriminate between individuals from different populations (see **Chapter 4**). Next to precopulatory sexual selection, the ability for long-time sperm storage from different mating partners, complex sperm storage organs and a mechanism for the digestion of excess sperm are known (Baur 1998, Beese *et al.* 2006, Beese *et al.* 2009), which and can influence the outcome of sperm utilization (Haase and Baur 1995, Chase and Darbyson 2008).

Additionally, genetic analyses indicated a low level of self-fertilization in one of the four populations observed. It is assumed that the ability to reproduce by self-fertilization is a means of coping with limited dispersal ability or low mate encounter rate in low-density populations (Levins 1968). However, no differences could be found in self-fertilization rate related to the population densities estimated. In *A. arbustorum*, self-

fertilizations seems to occur relatively seldom (see **Chapter 3** and **Chapter 4**) and/or only under rather harsh conditions (under isolation: Chen 1994, outbreeding: **Chapter 4**) possibly due to the consequences of lowered fitness of the selfed compared to outcrossed progeny (*i.e.* inbreeding depression; Frankham 1995, Auld and Relyea 2010).

In **Chapter 3**, the results showed that adult (experienced) snails used sperm received from subadult (young/unexperienced) mating partners for the fertilization of their eggs in the same frequency as sperm from adult mating partners. This indicated that the male function of snails with unfinished shells do not differ from that of adult snails. Furthermore, an unfinished shell could not be considered as a reliable indicator for virginity, because 35% of the subadult snails had already successfully mated prior to the copulation observed in the study. Matings between subadult and adult individuals have been observed in several hermaphroditic terrestrial gastropod species including *Theba pisana* (Cowie 1980), *A. arbustorum* (Baur 1984) and the nudibranch *Phestilla sibogae* (Todd *et al*. 1997). Furthermore, mate choice experiments revealed that snails do not discriminate between subadult and adult partners in *A. arbustorum* (E. Häussler, unpubl. data) and in *Lymnaea stagnalis* (Koene *et al*. 2008). The lack of precopulatory and postcopulatory mate choice with respect to mating history does not imply that there is no mate choice at all in *A. arbustorum*. Choice experiments revealed precopulatory discrimination between individuals from two distant populations of *A. arbustorum* in Switzerland, suggesting incipient reproductive isolation (Baur and Baur 1992*a*, **Chapter 4**). However, it seems that shell growth is not a discriminative factor for sexual selection in *A. arbustorum*. As it is observed in this study, subadult snails produced fewer eggs than adults, most probably because they still had to invest resources into growth. Consequently, the reduced number of offspring produced may counteract the evolutionary advantage of preferring a young partner with low sperm competition risk.

Sexual selection goes hand-in-hand with reproductive isolation and both can influence gene flow, genetic diversity and biodiversity. In **Chapter 4**, different mechanisms of reproductive isolation and sexual selection occurring between individuals from two distant populations of *A. arbustorum* were examined. We found that the number of eggs produced did not differ between homotypic and heterotypic matings. Consequently, reproductive compatibility is still maintained between

individuals of both populations. Furthermore, in **Chapter 4** sperm utilization was not random and highly skewed in the progeny of 44.4% of the double-mated snails. These non-random, highly skewed paternity patterns in the progeny could be a result of precopulatory and/or postcopulatory sexual selection, *e.g.* sperm competition or cryptic female choice (Birkhead and Møller 1998, see also above and **Chapter 2** and **Chapter 3**). On the other hand, our results indicated that the mating order seems to influence also, apart of the origin of the mating partner, sperm utilization pattern. However, this study also showed that a partial precopulatory isolation between individuals of the populations exists and mating partner are able to distinguish between partners from different populations. These differences may partly be population-specific because – within a population – individuals of *A. arbustorum* do not discriminate in respect to shell size, relatedness and mating history (Baur 1992, Baur and Baur 1997, E. Häussler unpubl. data, **Chapter 3**). Slight differences could exist in courtship behaviour, and/or in the composition of components in the snails' skin or in the mucus like allohormones, *i.e.* substances which induce direct effect without sensory identification, and pheromones. It has been demonstrated that allohormones and pheromones influence the mating process in various invertebrate species (Koene 2005). Similarly, mate choice experiments with individuals of the closely related land snails *Bradybaena pellucida* and *Bradybaena similaris* revealed that reproductive isolation was associated with differences in sexual pheromones (Wiwegweaw *et al.* 2009*a*). Furthermore, the genetic analyses of this study revealed a low frequency of self-fertilization (3.7% of all hatchlings) in the progeny of double-mated individuals of *A. arbustorum*. This result corresponds with the self-fertilization rate observed in natural populations (**Chapter 2** and **3**) and under lab conditions (Chen 1994). Overall, outcrossing even under outbreeding circumstances was preferred to self-fertilization as observed in this study. This result indicates that potential negative fitness consequences due to outbreeding depression are less costly than inbreeding depression as a result of self-fertilization (Frankham 1995, Wiwegweaw *et al.* 2009*b*).

As shown in the first chapters of this thesis, highly skewed sperm utilization patterns can be found in offspring arrays of individuals of *A. arbustorum*. The patterns observed are derived from parentage analyses, which reveal only the ultimate outcomes of the interactions between male and female reproductive processes (Schärer *et al.* 2007). However, to understand the patterns of sexual selection after copulation, the

mechanisms of sperm storage and sperm digestion are of relevant importance, beside of sperm utilization patterns. In **Chapter 5**, a novel technique to track the fate of sperm in the reproductive female organs of *A. arbustorum* is presented and tested. Our tests demonstrated a high efficiency of the sperm labelling procedure and show that labelled sperm can be visualized in the female reproductive organs of the receiver with a high reliability. Because of the wide use of the labelling substance in other invertebrate and vertebrate species (Moore *et al*. 1994, Zakharov *et al*. 1998, Gorbushin and Iankovleva 2006), the immunostaining technique presented should be transferable to other species used in sexual selection research. Following the fate of sperm from two or more males with this sperm-labelling technique could give insight into the mechanisms underlying sperm competition and cryptic female choice. The novel technique allows further investigations of the highly skewed sperm utilization patterns observed in the present thesis (**Chapter 2**, **3** and **4**) and it may improve our understanding of the evolution of male and female morphology and physiology in hermaphrodites and gonochorists.

OUTLOOK

In the present thesis, mate choice and sperm utilization patterns were investigated to gain further insight in the processes of sexual selection in the simultaneously hermaphroditic land snail *A. arbustorum*. Precopulatory sexual selection, *i.e.* mate choice, seems to be more pronounced in matings between individuals from different populations than in matings between individuals from the same population. There are several possible explanations for this pattern. First, it has been suggested that in simultaneous hermaphrodites, selection on traits related to mate acquisition is intrinsically weaker than in gonochorists and that the postcopulatory mechanism (*i.e.* female sperm manipulation) might be more essential (Greeff and Michiels 1999). Second, also population-specific differences in snail activity parameters during mating could lead to mate discrimination. Third, individuals from different populations are influenced by different environmental conditions which could alter chemical cues in the snail mucus or of the skin and would allow discrimination during courtship and mating due to the intensive body contact. Chemical analyses of substances in the snail mucus or skin and in individuals from different populations could provide further information about the mechanism underlying mate choice processes in *A. arbustorum*. Overall,

further studies are required to reveal the existing mechanisms in mate choice processes in this land snail species.

As mentioned above, postcopulatory sexual selection could have a high impact on mate selection in simultaneously hermaphroditic species. Skewed sperm utilization patterns were found in several studies in this thesis which indicates the occurrence of postcopulatory sexual selection in *A. arbustorum*. However, we used paternity estimates which only can reveal the ultimate outcomes of the interactions between male and female reproductive processes (Schärer *et al.* 2007). For the investigation of postcopulatory sexual selection, the novel sperm labelling technique presented in this thesis would allow further studies *e.g.* with double-mated individuals. This could help to enhance the understanding of the mechanistic principles of digestion, storage and utilization of competing sperm in the female reproductive organs of *A. arbustorum*.

In this thesis, many new facts about sexual selection processes in the simultaneously hermaphroditic land snail *A. arbustorum* could be revealed. However, many other questions have been opened and need further studies to shed light in the processes of precopulatory and postcopulatory sexual selection in simultaneous hermaphrodites.

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