# Selection on male and female reproductive traits in a simultaneous hermaphrodite land snail

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To *Arianta* who made it all possible.

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## Table of contents

# Summary

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. This thesis investigates sex allocation and precopulatory sexual selection by considering the influence of environmental conditions and behaviour on the reproductive allocation to the female and male function in the simultaneous hermaphrodite land snail *Arianta arbustorum* (L.). Furthermore, sperm competition and cryptic female choice are assumed to be crucial in determining fertilization success in this species because of the presence of multiple mating and long-term sperm storage. This work studies postcopulatory sexual selection mechanisms by considering sperm quality traits. Stylommatophoran gastropods have extraordinary long sperm. However, the extent of intra- and interindividual variation has rarely been examined.

First, we investigated the effects of soil type and adult size (shell volume) on mating propensity and female and male reproductive output (number and mass of eggs, number of sperm delivered and spermatophore mass) in individuals from two populations kept both on calcium-(Ca-)rich and Ca-poor soil. Independent of population and shell size, the mating propensity was higher and the total number of eggs produced was larger in snails kept on Ca-poor soil than in individuals reared on Ca-rich soil. We supposed that the Ca-poor soil used in the experiment still contained enough Ca to allow reproduction. Moreover, the Ca-rich soil could contain minerals or (unknown) substances which discourage reproduction in *A. arbustorum*.

In individual *A. arbustorum*, we assessed determinants of mating success and female and male reproductive success. 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. Our results challenge the trade-off assumption of sex allocation theory in simultaneous hermaphrodites. 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.

Sperm competition is one of the principal determinants of male fitness in species in which females mate promiscuously. The selective pressures it causes, though, are only partly understood, especially with respect to sperm characteristics favoured in sperm competition. We assessed among- and within-population variation in sperm length and number of sperm transferred in *A. arbustorum* from four natural populations. Sperm competition models on the evolution of sperm size assume associations with other sperm quality traits. Thus, we assessed variation in velocity, motility, and longevity of sperm in snails from two of the four populations. Independent of shell size, sperm length differed among populations and, to a minor extent, even among individuals within populations. Mean sperm length of a snail was not correlated with the number of sperm delivered in a spermatophore. The mean sperm velocity (=VCL) did not differ between snails from two populations. However, VCL varied among snails. Percentage motility and longevity of sperm differed between snails from the two populations. No correlations were found between length, velocity, percentage motility, and longevity of sperm. To conclude, individual snails differed in sperm quality, and this variation may partly explain the differential fertilization success between *A. arbustorum* snails. Moreover, our findings did not support the positive association between sperm length and longevity assumed by sperm competition models for internally fertilizing species.

The adaptive significance of sperm length variation is still unknown in *A. arbustorum*. Sexual selection on sperm length requires a significant additive genetic variance. Here we present the first estimates of narrow sense heritability of sperm length in this land snail. Sperm delivered by the same individuals in 2–4 matings over two reproductive seasons did not differ in length, indicating a high repeatability of this trait. Offspring of 10 families were kept at three temperatures (11, 15 and 20 °C) to examine the influence of different environmental conditions on sperm length and adult shell size. Sperm length was affected by temperature but not by family of origin, while adult shell breadth was influenced by temperature and family of origin. Higher temperatures resulted in shorter sperm but larger shells. The heritability of sperm length derived from the two different approaches (one-parent–offspring regression: $h^2 \pm SE = 0.52 \pm 0.55$; full-sibling split design: $H^2 \pm SE = -0.19 \pm 0.28$) suggests relatively little genetic variation in this trait in the studied population. In contrast, the heritability of adult shell breadth indicates a strong genetic effect (mother-offspring regression, $h^2 \pm SE = 0.90 \pm 0.33$). The heritability ($h^2 \pm SE$) of adult shell breadth obtained from the father–offspring regression was $0.18 \pm 0.42$, i.e. 5 times smaller than that of the mother–offspring regression, suggesting a maternal effect on shell size.

# General Introduction

Life histories are particularly suited to evolutionary analysis because the two major traits, survival and reproduction, are components of fitness (Silvertown & Doust, 1995). The fittest individuals in a population are, by definition, those that leave the greatest number of descendants (Begon *et al.*, 1990). Charles Darwin (1871) distinguished between "*natural selection*", in which individuals are selected according to their abilities to survive and reproduce in a particular habitat, and "sexual selection", in which they are selected according to their abilities to obtained more or better mates than other individuals (Silvertown & Doust, 1995). The life history favoured by natural selection from among those available in the population will be the one which has the highest total reproductive output, and depends on the habitat of the organism concerned (Begon *et al.*, 1990). Sexual selection promotes traits that confer an advantage in reproductive competition, in spite of being costly in the perspective of natural selection (e.g. the horns of horned beetles, or the fantastic plumes of the peacock tail; Møller, 1998).

Sexual selection operates through two fundamentally different mechanisms (Fig. 1; Pizzari & Parker, 2009): a) an intrasexual component of male-male competition for access to females, and b) an intersexual component of female selection of copulation partners. Thus, sexual selection may occur before mating (pre-insemination; Fig. 1). In most taxa, individual females may copulate (or spawn) with multiple males (i.e., are polyandrous). A consequence of polyandry is the potential for inter- and intrasexual selection to continue after copulation (post-insemination; Fig. 1). By means of controlled processes or structures, ejaculates of rival males may compete to fertilize the same set of eggs (sperm competition; Fig.1), and/or females may selectively favour paternity of males with a particular trait over that of other males (cryptic female choice or female sperm selection; Fig.1).

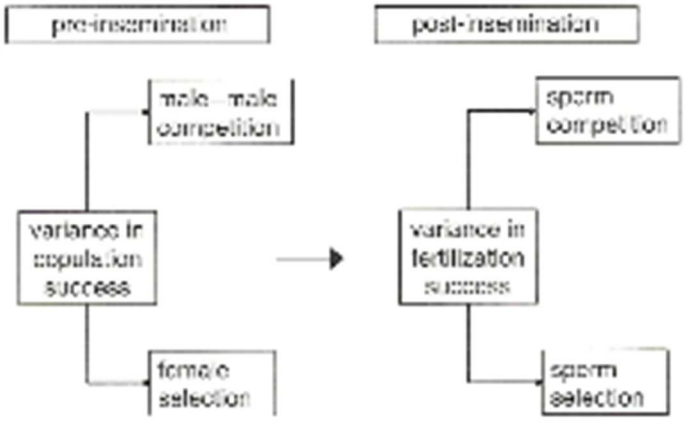


Figure 1. Diagrammatic representation of the main mechanisms of sexual selection (Pizzari & Parker, 2009)

In general, the study of sexual selection attempts to explain the evolution of structure and behaviour associated to reproduction (Birkhead & Møller, 1998). Michiels (1998) discussed the consequences of sexual selection and sperm competition in simultaneous hermaphrodites. Simultaneous hermaphroditism is widespread among plants and animals, and adaptations resulting from sexual selection are sometimes unique to this mating system. On the one hand, selection on male traits cannot be independent from the selection on female traits of the same individual. Thus, simultaneous hermaphrodites can optimize their reproductive allocation to the female and male function. On the other hand, conflicts between mating partners are particularly strong during copulation because of different mating interest. Indeed, conflicts arise within and between individuals that do not exist in gonochoristic species (Birkhead & Møller, 1998).

Reproductive resource allocation (or sex allocation) is a fundamental aspect of life history with profound ecological and evolutionary consequences in all sexual organisms (Stearns, 1992). In simultaneous hermaphrodites, sex allocation represents a decision about how resources are allocated to different organs and behaviours within an individual, given its reproductive mode, and given certain environmental and social conditions in which the organism lives (Schärer, 2009). The variable part of male and female investment is not restricted to gamete production. It may involve investments toward the production of seminal fluids, love darts, and egg shells, or toward the performance of sex-specific reproductive behaviours, such as mate searching, courtship, or egg laying (Schärer, 2009).

Factors thought to limit the fitness returns for allocation to male and female reproduction in hermaphrodites are to some extent linked to pre- and post-insemination sexual selection. For example, a female-biased sex allocation is favoured when the number of mates is limited (i.e. weak sperm competition), and a shift toward a more male-biased sex allocation is favoured with increasing numbers of mates (i.e. strong sperm competition). Such biological processes should be included in models for simultaneous hermaphrodites to make predictions on the shape of fitness gain curves and the resulting sex allocation patterns (Schärer, 2009). However, compared to gonochorists, quantitative data are needed that relate sexual selection and sex allocation in simultaneous hermaphrodites to the mating frequency, sperm precedence patterns, sperm displacement, sperm digestion, and cryptic female choice (Michiels, 1998; Schärer, 2009).

Sperm competition is one of the key processes in male-male competition (Fig. 1) and is defined as the competition between the sperm of two or more males for the fertilisation of a given set of ova (Parker, 1970). Adaptations to sperm competition occur at many biological levels (Parker, 1998): they may be behavioural (e.g. mate-guarding), physiological (e.g. male accessory gland fluid inducing unreceptivity after mating), and/or anatomical (e.g. copulatory plugs). Sperm competition studies investigate how a

male should allocate sperm among different ejaculates, or how he outdoes rival males. The evolution of sperm phenotype under sperm competition has received relatively little attention (Pizzarri & Parker, 2009). The mating or ejaculatory strategy which is the best for a male need not to be the best for the female. Thus, sperm competition can involve sexual conflict in which the interests of male and female differ (Parker, 1998). There is evidence of female control of sperm competition in some species, through either behavioural or physiological processes (Eberhard, 1996).

The aim of my thesis was to improve our understanding of sexual selection mechanisms in simultaneous hermaphrodites. Baur (1998) reviewed evolutionary aspects of sexual selection in molluscs: reproductive morphology, physiology, and behaviour that have implications for sperm competition. He concluded that not all groups of molluscs have received the same attention and that their potential as experimental organisms has not yet begun to be exploited by behavioural and evolutionary biologists. In particular, gastropods and cephalopods are unique because of several important features. Their elaborate mating behaviour may rival the complexity of those of various vertebrates (Baur, 1998).

## FOCUS OF THE THESIS

In this thesis I investigated different male and female aspects of sex allocation theory and studied sperm competition mechanisms in the simultaneously hermaphroditic land snail *Arianta arbustorum* (Linnaeus, 1758). This model organism fulfils the main prerequisites for sperm competition: (a) *A. arbustorum* mates repeatedly in the course of a reproductive season in the field, and (b) fertile sperm can be stored for more than 1 year (Baur, 1988). Additionally, 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). Multiple mating and sperm storage might enhance postcopulatory processes as competition among sperm from different partners, and/or selective storage and use of allosperm from the receiver (Baur, 1994a; Kupfernagel *et al.*, 2010). I asked the following main questions: Chapter I - how are resources allocated to different organs and behaviours within an individual given certain environmental conditions? Chapter II - how do behavioural and genetic traits influence mate choice, and female and male reproductive success? Chapter III - do snails differ in sperm quality characteristics that in turn may play a role in sperm competition? and finally Chapter IV - is there the potential for evolution to select individuals according to the length of their sperm?

Life-history theory predicts that a species occurring in different environments exhibits inter-populational variation in life-history traits as a result of different selection pressures (Stearns, 1992). However, observed local differences in life histories may also result from founder effects, genetic drift, and phenotypic plasticity (Calow, 1978; Caswell, 1983). Life-history variation may also be the result of

developmental plasticity or physiological acclimatisation. It follows that the observed variation in life history could simply mirror differences in habitat quality. It is well established that soil type and calcium availability influence shell growth and female reproductive output (number of eggs laid) in terrestrial gastropods (Baur, 1994b; Heller, 2001). Scarcity of Ca may result in thinner and more brittle shells (Voelker, 1959), rendering the snails less fit to protect the soft body properly against desiccation, physical damage, and invertebrate predators. In several species of terrestrial gastropods, Ca-provision to the eggs represents a major cost to the parent. The Ca concentration in *A. arbustorum* eggs may range from 5 to 8% of their dry mass (cf. Tompa, 1976). In **Chapter I,** we present the results of an experiment designed to examine whether mating propensity and sex specific reproductive allocation in *A. arbustorum* are affected by the type of soil. In a reciprocal transplant experiment, snails from habitats with Ca-rich and Ca-poor soils were kept either on their original soil or on the other soil under laboratory conditions. In particular, we asked whether the origin of the snails, adult shell size and/or soil type affected female and male reproductive output (number and mass of eggs, number of sperm delivered and spermatophore mass). We also examined whether the ratio of the resources allocated either to the male or female reproductive output is affected by the snails`origin, shell size, and soil type.

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). 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. In **Chapter II**, we observed the mating frequency and videorecorded the behaviour of individually tagged snails kept in groups of six animals over one reproductive season (58 days) under semi-natural conditions. 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.

Investment in sperm quality traits and in sperm number must be considered when examining the evolution of sperm characteristics through sperm competition. Sperm characteristics have so far been examined exclusively in gonochoristic species, with the exception of the hermaphroditic nematode *Caenorhabditis elegans* (LaMunyon & Ward, 2002). Sexual selection is also likely to shape sperm

characteristics in simultaneous hermaphrodites, even though this may conflict with the sperm receiver's interests (Michiels, 1998). The sperm donor must persuade the sperm receiver to use its sperm to fertilize eggs, and/or to avoid the postcopulatory control mechanisms (Michiels, 1998). In gastropods, interspecific differences in sperm morphology have been studied, while the intraspecific variation in sperm traits has not yet been analysed quantitatively. Sperm morphology is used as a taxonomical character (e.g. Healy, 1996). In taxa with sperm storage organs, sperm length may determine the ability to reach the storage organs first and to move to the ovum from the storage organ once ovulation takes place. Furthermore, within species, sperm–female interactions have been proved to be a major factor influencing sperm length evolution (e.g. Miller & Pitnick, 2002; Pattarini *et al*., 2006; Pitnick *et al*., 2009). In **Chapter III**, we assessed among- and within-population variation in sperm length and number of sperm transferred during copulation in *A. arbustorum* from four natural populations. To test the assumptions of sperm competition models on the evolution of sperm size (Parker, 1998), we measured the velocity, motility, and longevity of sperm, and we assessed their relationship with sperm length in two of the examined populations.

Different processes of postcopulatory sexual selection may result in sperm size differences (sperm competition, e.g. LaMunyon & Ward, 2002; cryptic female choice, e.g. Pitnick *et al.*, 2003), which, in turn, may lead to different paternity success (e.g. Oppliger *et al.*, 2003). Therefore, inter-individual differences in sperm length could account for the unexplained variance in fertilization success in *A. arbustorum* (Baur, 1998). Sexual selection on sperm length requires a significant additive genetic variance, but few studies have actually measured this. In **Chapter IV**, we present the first estimates of heritability of sperm length in the land snail *Arianta arbustorum* (L.) using two complementary approaches (one-parent–offspring regression and full-sibling split design). We also examined whether sperm length is influenced by the shell size of the snail and estimated heritability of shell size.

Finally, in the section "**General Discussion**", an overview of the results is given, as well as a discussion on how this thesis contributes to a better understanding of complex behaviour and reproductive strategies in hermaphrodites.

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# Chapter I

## Effects of soil type and adult size on mating propensity and reproductive output in two populations of the land snail *Arianta arbustorum* (Linnaeus)

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# EFFECTS OF SOIL TYPE AND ADULT SIZE ON MATING PROPENSITY AND REPRODUCTIVE OUTPUT IN TWO POPULATIONS OF THE LAND SNAIL *ARIANTA ARBUSTORUM* (LINNAEUS)

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## ABSTRACT

Life-history traits in terrestrial gastropods may be influenced by both abiotic and biotic factors. This study examines the effects of soil type and adult size (shell volume) on mating propensity and female and male reproductive output (number and mass of eggs, number of sperm delivered and spermatophore mass) in individuals of the simultaneous hermaphrodite land snail *Arianta arbustorum* from two populations kept both on calcium-(Ca-)rich and Ca-poor soil. Snails from the two populations differed in adult size, relative shell growth, mating propensity and egg size. Furthermore, in both populations the number of egg batches deposited, egg size and spermatophore size scaled allometrically with shell volume, but not the total number of eggs produced and number of sperm delivered. Independent of population and shell size, the type of soil on which the snails were maintained influenced mating propensity, the total number of eggs produced and the mass of the albumen gland (another measure of female reproductive output). The mating propensity was higher and the total number of eggs produced was larger in snails kept on Ca-poor soil than in individuals reared on Ca-rich soil. This surprising finding could be explained by the fact that the Ca-poor soil used in the experiment still contained enough Ca to allow reproduction, and that the snails ingested Ca through the food consumed (lettuce grown on Ca-rich soil was available *ad libitum*). Moreover, the Ca-rich soil could contain minerals or (unknown) substances which discourage reproduction in *A. arbustorum*. Our study highlights the complexities faced when interpreting differences in the life history of gastropods. Explaining interpopulational differences in life-history patterns requires not only the understanding of the influence of snail origin, but also an understanding of the effects of shell size, substratum type (soil type), food and local climate.

Key words: *Arianta arbustorum*, calcium availability, egg size, reproductive allocation, simultaneous hermaphrodite, sperm number.

## INTRODUCTION

Life-history theory predicts that a species occurring in different environments exhibits interpopulational variation in life-history traits as a result of different selection pressures (Stearns, 1992). However, observed local differences in life histories may also result from founder effects, genetic drift, and phenotypic plasticity (Calow, 1978; Caswell, 1983). Moreover, evolutionary interpretations of life-history patterns require a distinction to be made between genotypic and environmentally induced phenotypic variation, because life-history variation may also be the result of developmental plasticity or physiological acclimatization. It follows that observed variation in life history could simply mirror differences in habitat quality.

Reproductive resource allocation is a fundamental aspect of life history with profound ecological and evolutionary consequences (Stearns, 1992). Shelled gastropods strongly depend on calcium (Ca) as a major macronutrient constituent of their body (Dallinger et al., 2001). Besides reinforcing the shell, Ca is critical to a variety of functions in soft-tissue metabolism and reproduction (Tompa & Wilbur, 1977; Porcel et al., 1996). Most of the Ca absorbed by terrestrial gastropods may enter the animal's body via the epithelium of the intestine (Dexheimer, 1963; Beeby & Richmond, 2007). Because of seasonal needs for Ca, snails store

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this mineral mainly as calcium carbonate and possess high reallocation capacities (Fournié & Chétail, 1984). Ca can be mobilized readily from the intracellular storage site during periods of increased demand (Tompa & Wilbur, 1977). Ca provision to the eggs represents a major cost to the parent, and a variety of strategies are used to ensure sufficient Ca for the hatchling to build a shell (Tompa, 1980; Baur, 1994a). It is well established that soil type and calcium availability influence shell growth and female reproductive output (number of eggs laid) in terrestrial gastropods (Baur, 1994a; Heller, 2001). For example, egg production of *Helix pomatia* Linnaeus, 1758, exposed to acid soil was doubled when calcium carbonate was supplied during an experiment (Crowell, 1973). However, the potential effect of soil type on mating behaviour and the male reproductive output (spermatophore size and number of sperm delivered during copulation) have not been investigated in terrestrial gastropods.

Here we present the results of an experiment designed to examine whether mating propensity and sex-specific reproductive allocation in the simultaneous hermaphrodite land snail *Arianta arbustorum* (Linnaeus, 1758) are affected by the type of soil. In a reciprocal transplant experiment, snails from habitats with Ca-rich and Ca-poor soils were kept either on their original soil or on the other soil under laboratory conditions. In particular, we asked whether the origin of the snails, adult shell size and/or soil type influence the mating propensity, number of sperm delivered during copulation, and number of eggs laid within a season in *A. arbustorum*. We also examined whether the ratio of the resources allocated either to the male or female reproductive output is affected by the snails' origin, shell size, and soil type.

## MATERIALS AND METHODS

### Study Animals

*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 & Raboud, 1988; Baur, 1990). In contrast to male fecundity (i.e., sperm expenditure), female fecundity (i.e., clutch size and the number of batches produced per season) is positively correlated with adult shell size (Baur, 1994a; Baur et al., 1998).

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 (Hofmann, 1923; Baur, 1992a). Copulation is reciprocal; after intromission, each snail transfers simultaneously 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 one year (Baur, 1988). Mating is random with respect to shell size and different degrees of relatedness (Baur, 1992a; Baur & Baur, 1997). Individuals need at least eight days to replenish their sperm reserves after a successful copulation (Locher & Baur, 1999; Hänggi et al., 2002).

Paternity analysis in broods of wild-caught *A. arbustorum* showed a high frequency of multiple insemination (Baur, 1994b). A controlled laboratory experiment showed that one successful copulation per reproductive season is sufficient to fertilize all the eggs produced by an individual (Chen & Baur, 1993). However, 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 & Baur, 1993).

*Arianta arbustorum* feeds on vascular plants and dead or senescent herbs, but the snails often supplement their vegetable diet with microorganisms, soil and carrion (Frömming, 1954; Grime & Blythe, 1969).

### General Methods

Subadult *A. arbustorum* were collected from a locality with Ca-rich soil (Gurnigel) and a locality with Ca-poor soil (Zastler) in late April and early May 2002. Snails in the site with Ca-rich soil inhabited the embankment of a track in the subalpine forest near Gurnigel, 30 km south of Bern, Switzerland (46°45′N, 7°27′E; elevation 1,320 m above sea level), those in the site with Ca-poor soil the embankment of a forest road in the Black forest, Germany (47°54′N, 8°01′E; elevation 1,060 m). The two sites were 130 km apart.

Topsoil from both localities was collected as substrata for snail maintenance. Three addi-

TABLE 1. Results of the chemical soil analyses. See text for further explanation of variables.

| Soil parameter | Gurnigel, Ca-rich soil | Zastler, Ca-poor soil |
|---|---|---|
| Calcium (Ca)* | 1,409 | 99 |
| pH ($H_2O$) | 7.2 | 5.8 |
| Total nitrogen (%) | 0.37 | 0.30 |
| C/N-ratio | 11.5 | 15.2 |
| Phosphorus ($P_2O_5$)* | 0.2 | 1.3 |
| Potassium ($K_2O$)* | 19 | 17 |
| Magnesium (Mg)* | 6 | 17 |
| Copper (Cu)* | 0.03 | 0.24 |
| Iron (Fe)* | 0.6 | 18.0 |
| Manganese (Mn)* | 0.3 | 9.4 |
| Zinc (Zn)* | 0.1 | 1.7 |

*in mg/100 g dry soil

tional samples from each site were combined for soil analyses: the percentage of nitrogen (N), the content (in mg per 100 g dry soil) of calcium (Ca), phosphorus ($P_2O_5$), potassium ($K_2O$), magnesium (Mg), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn), the C/N-ratio and the soil pH in water. The soil samples were analysed using standard protocols by the laboratory of F. M. Balzer, Wetter-Amönau, Germany. Characteristics of the two soils are summarized in Table 1. The Ca content represents both the exchangeable calcium and the calcium carbonate content of the soil.

The snails were kept isolated in transparent beakers (8 cm deep, 6.5 cm in diameter) lined with moist soil (approximately 4 cm) outdoors at a shaded place. Fresh lettuce was provided twice per week and at the same time the beakers were cleaned. Snails from both sites were randomly assigned to one of two treatment groups. Half of the snails were raised on soil from their original locality, the other half on soil from the other locality (Gurnigel: 53 individuals on Ca-rich [original] soil, 52 individuals on Ca-poor soil; Zastler: each 51 individuals on Ca-poor [original] and Ca-rich soil).

Within four weeks, all individuals reached sexual maturity as indicated by the formation of a flanged lip at the shell aperture. The snails were marked individually with letters and numbers written 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.

Shell breadth of each snail was measured twice to the nearest 0.1 mm using vernier callipers immediately after being collected as subadults and after shell growth was completed. Relative shell growth of an individual was expressed by the difference between the two measurements divided by the initial size. In adult snails, shell height was also measured and shell volume was calculated using the formula: shell volume = 0.312 x [(breadth)$^2$ x height] – 0.038 (measurements in mm; B. Baur, unpublished data). Shell volume is a more reliable measurement of snail size than weight, because weight depends on the state of hydration and thus is highly variable in terrestrial gastropods.

Sexually mature snails were allowed to mate outdoors. Active snails (individuals with an extended soft body and everted tentacles) from each treatment group were placed in a transparent plastic container, measuring 25 x 18 x 7 cm, lined with moistened paper towelling. When a pair had started to court the snails were transferred to a smaller plastic container (14 x 10 x 7 cm) to allow mating to take place without disturbance from other non-courting snails. Mating trials were initiated in the evening and ran during three nights in June 2002. The period between the end of May and the middle of July is the time of maximum mating activity in subalpine populations of *A. arbustorum* (Baur, 1992a).

We observed the snails' courtship behaviour at intervals of 15 min (at night using a torch) following the method described in Baur (1992a) and recorded courtship duration (time interval from courtship initiation to copulation). Observation sessions were terminated either after successful copulation or after 14 h if no snail initiated courtship behaviour. Snails that did not mate were tested again seven days later with a new composition of snails within the same treatment group. Between two trials, snails of each group were maintained as described above.

After copulation, one randomly chosen mating partner (hereafter referred to as focal snail) was kept in a beaker as in the premating phase, but at 19°C with a light:dark cycle of 16:8 h. The other mating partner (hereafter referred to as sperm receiver) was frozen immediately after copulation. To obtain the spermatophore from the focal snail, we dissected out the female reproductive tract of the receiver. We measured the length (*L*) and width (*W*) of the sperm-containing part of each spermatophore to the nearest 0.1 mm using a dissecting microscope. Spermatophore size (in mm$^3$) was

approximated, by the formula ($\pi LW^2/4$), assuming a cylindrical volume. Spermatophores were kept singly in Eppendorf tubes at -30°C until sperm were counted.

The beakers of focal snails were checked 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 separated from remaining unhatched eggs to prevent egg cannibalism (Baur, 1992b). In all treatment groups eggs were collected over a period of 60 days following copulation. The length of this period corresponds to approximately one reproductive season of *A. arbustorum* living in the wild (Baur, 1990).

### Sperm Counting Procedure

We assessed the number of sperm that a focal snail delivered to its mating partner by counting the number of sperm in the spermatophore transferred. This procedure is described in detail in Locher & Baur (1997). The spermatophore of *A. arbustorum* consists of a hardened secretion which encapsulates the spermatozoa (Hofmann, 1923). We mechanically disrupted the spermatophore in 200 µl PBS-buffer (138.6 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \times 2H_2O$ and 1.5 mM $KH_2PO_4$) using a pair of microscissors. The sperm suspension was homogenized with a set of Gilson pipettes for 5–15 min. To count the sperm, we stained the homogenate for 1–3 h with an equal volume of a gallocyanin-chromium complex, which stains the DNA in the head of the spermatozoa. If spermatozoa still occurred in clusters, we treated the sample overnight with a sonicator (35 kHz). Two subsamples of known volume of the sperm suspension were diluted 1:3 with PBS-buffer and transferred to a Bürker-Türk counting chamber. This counting chamber consists of 16 cells each with a volume of 25 nl. We counted all sperm heads in randomly chosen cells until the total number of sperm heads exceeded 400, and used the average of two subsamples to calculate the total number of sperm in a spermatophore.

### Estimate of Sex-Specific Reproductive Allocation

As measures of female reproductive allocation the dry mass of all eggs produced by an individual and the dry mass of the albumen gland were considered. We assessed the dry weight of 48 randomly sampled eggs (three eggs each from eight snails from both populations, equally distributed over the soil types), calculated the mean dry weight of an egg for both populations on each soil type, and multiplied this by the number of eggs produced by each snail. At the end of the experiment, the albumen gland of each focal snail was dissected out of the female reproductive duct. We determined the dry mass of the albumen gland to the nearest 0.1 mg. The dry mass of spermatophores filled with spermatozoa was considered as a measure of male reproductive allocation. We used the relationship between the size of the spermatophores ($X$ = volume of the sperm container in $mm^3$) and the dry mass of spermatophores filled with spermatozoa ($Y$ in mg)

$$\ln(Y) = 0.670 \times \ln(X) - 0.524$$

to calculate the dry mass of the spermatophores produced by individual snails in these experiments (relationship from Locher & Baur, 2000).

### Data Analyses

The StatView program package (SAS Institute, 1998) was used for statistical analyses. Means ± 1 S.E. are given unless otherwise stated. Data which did not fit normal distributions were $\log_{10}$-transformed and frequency data (hatching success) were arcsine-transformed. Differences in shell size and relative growth rate between treatment groups (soil type) and populations were examined using two-way analysis of variance. However, allometric relationships may confound interpretations of differences in observed reproductive output between treatment groups and/or populations. To examine possible differences in reproductive traits analysis of covariance with soil type and population as factors and shell size as covariate was used (ANCOVA, type III model).

## RESULTS

### Shell Growth and Adult Size

At the beginning of the experiment, subadult snails from the Gurnigel population were smaller than those from the Zastler population (shell breadth: 15.0 ± 0.1 mm vs. 17.9 ± 0.1 mm; $F_{1,120} = 324.24$, $p < 0.0001$). However, subadult *A. arbustorum* from either population kept both

on Ca-rich or Ca-poor soil did not differ in shell breadth ($F_{1,120} = 0.0001$, $p = 0.99$).

All snails attained adult size within four weeks. Fully grown snails from the two populations differed in adult size (shell breadth: Gurnigel: 17.3 ± 0.1 mm; Zastler: 19.4 ± 0.1 mm; $F_{1,121} = 124.59$, $p < 0.0001$). In both populations, adult shell size was not affected by the type of soil on which the snails were kept ($F_{1,121} = 1.70$, $p = 0.20$). Relative shell growth differed among snails from the two populations (Gurnigel: 0.155 ± 0.008; Zastler: 0.084 ± 0.006; $F_{1,120} = 53.92$, $p < 0.0001$) and was slightly, but not significantly higher in snails kept on Ca-rich soil than in snails kept on Ca-poor soil ($F_{1,120} = 2.65$, $p = 0.106$).

## Mating Propensity

Copulations were observed in 75 (34.9%) out of 215 trials. Snails from the Gurnigel population showed a slightly but not significantly higher mating propensity (40.4%; 40 matings out of 99 trials) than those from the Zastler population (30.2%; 35 matings out of 116 trials; $\chi^2 = 2.46$, $df = 1$, $p = 0.13$). However, the type of soil affected the mating propensity. Independent of origin, snails kept on Ca-poor soil showed a higher mating propensity than snails kept on Ca-rich soil (43.9% vs. 27.4%; $\chi^2 = 6.41$, $df = 1$, $p = 0.015$, data from both populations combined). In the Zastler population, a larger proportion of snails kept on Ca-poor soil copulated compared with snails kept on Ca-rich soil (39.6% vs. 22.6%; $\chi^2 = 3.92$, $df = 1$, $p = 0.048$). Similarly, in the Gurnigel population, there was a tendency that more snails kept on Ca-poor soil copulated than snails kept on Ca-rich soil (50.0% vs. 33.3%; $\chi^2 = 2.79$, $df = 1$, $p = 0.08$). The time from initiation of courtship to copulation ranged from 6.5 to 24.5 h (mean: 12.3 h, n = 69) and was neither influenced by the type of soil ($F_{1,65} = 0.04$, $p = 0.84$) nor did it differ between the two populations ($F_{1,65} = 0.12$, $p = 0.73$).

## Female Reproductive Output

Snails deposited their first egg batch 10 to 39 days after copulation (mean: 13.1 days, n = 69). The time elapsed between copulation and first oviposition was neither influenced by the type of soil ($F_{1,65} = 0.19$, $p = 0.67$) nor did it differ between populations ($F_{1,65} = 0.01$, $p = 0.99$).

Snails from either population did not differ in number of egg batches produced (Table 2). However, the number of egg batches produced was affected by soil type (Table 3). Surprisingly, snails kept on Ca-poor soil deposited more batches than those maintained on Ca-rich soil (Table 2). Furthermore, the number of egg batches laid was influenced by shell size (Table 3). Similarly, the total number of eggs produced by each snail was affected by soil type (Table 3). Snails kept on Ca-poor soil produced more eggs than snails kept of Ca-rich soil (Table 2). The total number of eggs produced was not

TABLE 2. Female and male reproductive traits of *A. arbustorum* from two populations (Gurnigel and Zastler). Snails were kept both on Ca-rich soil (original substratum in Gurnigel) and Ca-poor soil (original substratum in Zastler). Mean values ± 1 S.E. with sample size in parentheses are presented. Results of ANCOVAs are shown (for details of statistical analyses, see Table 3).

| Trait | Gurnigel population | | Zastler population | | Effect of | | |
|---|---|---|---|---|---|---|---|
| | Ca-rich | Ca-poor | Ca-rich | Ca-poor | shell size | population | soil type |
| Number of egg batches | 3.7 ± 0.5 (15) | 4.0 ± 0.3 (21) | 3.8 ± 0.4 (14) | 4.5 ± 0.3 (19) | * | ns | * |
| Total number of eggs | 116 ± 14 (15) | 129 ± 12 (21) | 139 ± 15 (14) | 167 ± 11 (19) | ns | ns | * |
| Egg mass (mg) | 2.45 ± 0.09 (12) | 2.18 ± 0.04 (12) | 2.34 ± 0.14 (12) | 2.44 ± 0.10 (12) | * | ** | ns |
| Number of sperm delivered (x $10^3$) | 2194 ± 340 (15) | 2035 ± 276 (20) | 2705 ± 450 (13) | 2049 ± 230 (19) | ns | ns | ns |
| Spermatophore size, (volume of sperm container, $mm^3$) | 1.95 ± 0.21 (15) | 1.75 ± 0.15 (20) | 2.68 ± 0.28 (14) | 2.21 ± 0.19 (19) | * | ns | ns |

*$p < 0.05$, **$p < 0.01$, ns = not significant

affected by shell size (Table 3). In contrast, egg dry mass differed between populations and was significantly related to shell size, but was not affected by soil type (Table 3). Furthermore, the analysis of covariance had a significant interaction term (shell size x population), indicating differing slopes between populations (Table 3). In snails from the Zastler population, egg dry mass was positively related to the shell size of the mother snail ($R^2 = 0.52$, $n = 8$, $p = 0.0424$), whereas in the Gurnigel population no significant relationship was found ($R^2 = 0.08$, $n = 8$, $p = 0.49$).

Hatching success of eggs differed slightly, but not significantly between populations (Gurnigel: 82.3 ± 2.6%, Zastler: 75.3 ± 3.4%; $F_{1,65} = 3.62$, $p = 0.0617$), but was not affected by soil type ($F_{1,65} = 2.46$, $p = 0.12$).

Female reproductive output, represented by the dry mass of all eggs produced by an individual snail, ranged from 21 to 614 mg (mean: 327 mg, n = 69). Female reproductive output was affected by soil type (Ca-rich soil: 299 ± 25 mg; Ca-poor soil: 347 ± 21 mg; $F_{1,61} = 5.14$, $p = 0.0270$), but did not differ between the populations ($F_{1,61} = 0.25$, $p = 0.62$) and was not influenced by snail size ($F_{1,61} = 0.17$, $p = 0.68$). There was, however, a significant interaction term (shell size x soil type), indicating that shell size influenced female reproductive output on different soils in a different way ($F_{1,61} = 4.92$, $p = 0.0302$). The dry weight of the albumen gland, another measure of female reproductive output, ranged from 8.7 to 59.4 mg (mean: 29.5 mg, n = 66). As the dry mass of all eggs, the mass of the albumen gland was affected by soil type ($F_{1,58} = 4.45$, $p = 0.0392$), but no effects of population and shell size were found ($F_{1,58} = 0.87$, $p = 0.36$ and $F_{1,58} = 0.21$, $p = 0.65$).

TABLE 3. Analyses of covariance (ANCOVA) of the relationship between reproductive characters and shell size (volume, log-tranformed) of *A. arbustorum*. Snails from two populations were kept each on two soils (only significant interactions are shown).

| Dependent variable | Independent variable, Effects | df | Type III SS | F | p |
|---|---|---|---|---|---|
| Number of egg batches (log) | Shell size | 1 | 0.231 | 6.44 | 0.0137 |
| | Population | 1 | 0.035 | 0.97 | 0.33 |
| | Soil type | 1 | 0.182 | 5.07 | 0.0279 |
| | Shell size x soil type | 1 | 0.167 | 4.66 | 0.0347 |
| | Error | 61 | | | |
| Total number of eggs | Shell size | 1 | 518.622 | 0.18 | 0.68 |
| | Population | 1 | 635.611 | 0.22 | 0.64 |
| | Soil type | 1 | 15101.587 | 5.14 | 0.0269 |
| | Shell size x soil type | 1 | 14582.744 | 4.97 | 0.0295 |
| | Error | 61 | | | |
| Egg mass (log) | Shell size | 1 | 0.018 | 10.27 | 0.0125 |
| | Population | 1 | 0.023 | 13.53 | 0.0062 |
| | Soil type | 1 | 0.001 | 0.58 | 0.47 |
| | Shell size x population | 1 | 0.017 | 10.02 | 0.0133 |
| | Error | 8 | | | |
| Number of sperm delivered (log) | Shell size | 1 | 0.173 | 1.64 | 0.21 |
| | Population | 1 | 0.051 | 0.48 | 0.49 |
| | Soil type | 1 | 0.00003 | 0.003 | 0.99 |
| | Error | 59 | | | |
| Spermatophore size, | Shell size | 1 | 0.077 | 5.53 | 0.0220 |
| (volume of sperm container, log) | Population | 1 | 0.0001 | 0.01 | 0.92 |
| | Soil type | 1 | 0.002 | 0.13 | 0.72 |
| | Error | 60 | | | |

Male Reproductive Output

Spermatophore size, indicated by the volume of sperm container, was neither influenced by soil type nor by population (Table 3). However, spermatophore size was influenced by shell size (Table 3); it was positively correlated with shell volume ($r = 0.42$, $n = 68$, $p = 0.0003$). The number of sperm delivered in a spermatophore ranged from 192,000 to 6,026,000 (mean: 2,204,600, n = 67). Sperm number was neither affected by soil type, nor by the origin of the snails (population) and shell size (Tables 2, 3).

The male reproductive output, expressed as dry mass of both spermatozoa and spermatophore, ranged from 0.31 to 1.83 mg (mean: 0.96 mg, n = 68). Male reproductive output was affected by shell size ($F_{1,60} = 5.01$, $p = 0.0289$), but not by soil type and population ($F_{1,93} = 0.05$, $p = 0.83$ and $F_{1,60} = 0.11$, $p = 0.74$).

The relative allocation to the male reproductive function, expressed as percentage of the total dry mass devoted to the male function, ranged from 0.1 to 3.2% (mean = 0.4%, n = 68). The remaining 96.8 to 99.9% of the dry mass were allocated to the female reproductive output. The relative allocation to the male reproductive function was neither influenced by soil type, population nor by shell size (in all cases $p > 0.20$).

## DISCUSSION

The present study showed that the type of soil can affect the mating propensity and female reproductive output in a simultaneous hermaphrodite land snail. Mating propensity and other life-history traits including adult shell size, shell growth, and egg size were also influenced by the snails' origin, and still others (egg size, number of egg batches produced and spermatophore size) were affected by shell size. Effects of soil type on reproductive traits have so far received little attention in terrestrial gastropods. The breeding behaviour of *Cernuella virgata* (da Costa, 1778) was affected both by soil type and soil moisture content, whereas the total number of eggs laid and the tendency to lay the first egg were influenced by soil type (Carne-Cavagnaro et al., 2006). Juveniles of *Cornu aspersum* (Müller, 1774) from different sites exhibited differential growth rates and Ca-allocation strategies (Beeby & Richmond, 2007). Land snails may be able to assess the Ca concentration in their food plants and in the soil (Chevalier et al., 2003) and may actively balance their diet to optimise the Ca intake (Iglesias & Castillejo, 1999). Soil constitutes a key component of the snails' diet in natural populations. In *C. aspersum*, hatchlings which fed on soil outgrew other siblings, indicating the importance of certain soil characteristics and of exchangeable Ca (Gomot et al., 1989).

In a laboratory experiment, the Ca concentration did not affect shell growth and adult size in subadult individuals of *A. arbustorum* reared on agar-based diets with different levels of Ca (Wacker & Baur, 2004). Snails reared on intermediate- and low-calcium diets increased their consumption rates, but despite compensatory feeding, these snails were unable to take up the amount of Ca required for metabolism and shell growth and thus had a higher mortality.

The Ca-provision to the eggs represents a major cost to the parent. Among gastropods, a variety of strategies are used to ensure sufficient Ca to the eggs (Tompa, 1980; Baur, 1994a). Ca is used for the calcification of the embryonic shell and for the deposition of Ca reserves which differentiate during the embryonic life (Fournié & Chétail, 1984). The Ca requirements for embryonic life explain the considerable amount of Ca that the adult gastropod looses during the oviposition period. For example, *Anguispira alternata* (Say, 1816) mobilizes 10–25 mg Ca for one egg batch in less than a day (Tompa, 1975).

In several species of terrestrial gastropods, a significant amount of Ca is embedded in the egg shell. *Arianta arbustorum* has partly calcified eggs with discrete crystals of calcium carbonate in a jelly matrix. Compared with species that produce similar eggs, the Ca concentration in *A. arbustorum* eggs may range from 5 to 8% of their dry mass (cf. Tompa, 1976). Thus, snails kept on Ca-poor soil may have invested 17–28 mg Ca into the eggs produced in the course of the experiment, those kept on Ca-rich soil 15–24 mg Ca. The Ca-content of lettuce varies between 300 and 500 mg/kg (Chevalier et al., 2003). Individual *A. arbustorum*, kept under similar experimental conditions as in the present study, consumed a leaf area of 32–50 $cm^2$ lettuce per week, which corresponds to a Ca-uptake of 0.26 to 0.68 mg per week (Locher & Baur, 2002). The assimilation efficiency of consumed lettuce averages 97% in *A. arbustorum* (dry mass; Abdel-Rehim, 1987). Considering the entire experimental period of 88 days, this resulted in a Ca-uptake of 3.3–8.5 mg, indicating that a considerable amount of Ca invested in egg

production was actually obtained from the lettuce consumed. Thus, snails kept on Ca-poor soil could partly compensate the Ca-deficiency in the soil by consuming more lettuce. It should be noted, however, that the experimental conditions in our study did not represent the natural situation, because plants growing on Ca-poor soils usually have a low Ca-content. The same might be true for decaying plant material and leaf litter on Ca-poor soil.

Scarcity of Ca may result in thinner and more brittle shells (Voelker, 1959), rendering the snails less fit to protect the soft body properly against desiccation, physical damage and invertebrate predators. *Arianta arbustorum* shows a tolerance to acidic soils with low Ca availability (Kerney & Cameron, 1979). In the wild, however, adult *A. arbustorum* with more robust shells were found at the Ca-rich site at Gurnigel than at the Ca-poor site at Zastler (B. Baur, unpubl. data).

Because snails require substantial amounts of Ca for reproduction (Crowell, 1973; Wäreborn, 1979), growth (Gomot et al., 1989; Ireland, 1991), shell production, and metabolism (Fournié & Chétail, 1984), Ca availability is a key determinant of habitat quality in molluscs. In fact, greater abundance, species richness, and biomass of snails are generally observed on soils rich in Ca compared with soils poor in Ca (Boycott, 1934; Wäreborn, 1992; Hotopp, 2002; Ondina et al., 2004). Ca addition to mitigate effects of acid deposition resulted in a significant increase in snail abundance (Gärdenfors, 1992; Skeldon et al., 2007), whereas slugs did not show a similar pattern. However, processes other than acid deposition also influence Ca cycling and availability in soils. For example, wood succession resulting in a shift in the dominant tree species can change the foliar Ca concentration.

In suboptimal habitats where conditions are outside the range to which most snail individuals are adapted, some cost, expressed either as a reduction in growth, survival, or reproduction, may occur. However, apart from Ca availability, other soil parameters, such as soil physical properties (soil texture, moisture retention and chemistry) and availabilities of nutrients such as aluminium, nitrogen and magnesium, may influence the reproductive output of *A. arbustorum*. Soil type seems to be a key factor in the reproductive biology of *A. arbustorum* and most probably in other land snail species. However, it remains unclear which soil characteristics are important.

In our study, the total number of eggs produced was larger in snails kept on Ca-poor soil than in individuals maintained on Ca-rich soil. There are different possible explanations for this surprising finding: (1) the Ca-poor soil used in the experiment still contained enough Ca to allow egg production; (2) the snails ingested Ca from the lettuce offered and with compensatory feeding they partly counter-balanced the Ca deficiency; (3) the Ca-rich soil could contain minerals or (unknown) substances that disturbed reproduction in *A. arbustorum*; (4) the Ca-poor soil had other essential constituents for successful reproduction that were in short supply in the Ca-rich soil; (5) snails living under environmental stressing conditions (i.e., on Ca-poor soils) put more resources into reproduction in their first breeding but have a shorter lifespan than snails in more favourable conditions. The range of Ca content in different soil types is much wider in nature than in the two soils used in this experiment. Our Ca-poor soil still contained a low level of exchangeable Ca (Table 1). The hypothesis of compensatory feeding cannot be tested because the amount of lettuce consumed by each snail was not recorded. However, the albumen gland of snails kept on Ca-poor soil was larger than that of snails maintained on Ca-rich soil. This indicates that the former group ingested more food. In stylommatophoran gastropods, the albumen gland synthesizes the perivitelline fluid to be added to eggs for provisioning the developing embryo (Gomez, 2001). The size of this gland determines the maximum number of eggs that can be produced at any one time (Tompa, 1984). Most probably, snails kept on Ca-rich soil consumed for some unknown reasons less lettuce, which resulted both in a decreased mating propensity and reproductive output. Hatching success of eggs produced by snails kept on either soil type did not differ, suggesting that there was no difference in egg quality between the two treatment groups. At high concentrations, zinc, copper, lead and cadmium all negatively affect reproduction in *C. aspersum* (Laskowski & Hopkin, 1996). At low concentrations, however, no effect of these metals was recorded (Gomot, 1997). In contrast, other soil constituents may positively affect snail reproduction. For example, the Ca-poor soil contained three times more magnesium than the Ca-rich soil (Table 1). Unfortunately, besides Ca relatively little is known on the beneficial value of various soil constituents for land snail reproduction (Speiser, 2001). There may also

be a trade-off between reproductive output and survival. Snails living in environmentally stressing conditions may allocate more resources into reproduction in the first breeding season but may die earlier than those living in more favourable conditions. This hypothesis could be tested by maintaining snails over two or more years under the experimental conditions of the present study. We assume that a combination of different factors contributed to the unexpected result.

The present study examined to our knowledge for the first time soil-type related effects on male reproductive output. However, neither the number of sperm delivered nor spermatophore size differed between the two snail groups kept on different soils. Of the total reproductive output (expressed as dry mass), only 0.1–3.2% were devoted to the male function in form of sperm and spermatophore, while the remaining 96.8–99.9% were allocated to the female function in form of eggs. These values are very similar to previous estimates of male and female reproductive allocation in *A. arbustorum* (Locher & Baur, 2000, 2002).

In our study, several life-history traits were affected by the origin of the snails and/or their adult size. Size-related fecundity has been found in a variety of gastropods as well as in other invertebrates (Baur, 1998). In many land snail species, both clutch size and egg size are positively correlated with shell size (Heller, 2001). In the present study, the number of egg batches deposited, egg mass and spermatophore size were related to adult size in *A. arbustorum*.

In land snails, limited dispersal capacity and restricted habitat requirements may enhance the process of adaptation to local conditions. Genetic differentiation between populations of terrestrial gastropods has been demonstrated in several species (e.g., Jones et al., 1977; Clarke et al., 1978; Fearnley, 1996; Beeby & Richmond, 2001, 2007). The adult size of *A. arbustorum* decreases with increasing altitude in the Alps, whereas shell colour varies with the type of habitat (Burla & Stahel, 1983; Baur, 1984; Gosteli & Burla, 1993). Snails from different populations exhibit differences in resting site preference and geotactic response (Baur, 1986; Baur & Gosteli, 1986), as well as in such reproductive characters as clutch size and egg size (Baur & Raboud, 1988). Snails from the two populations examined in the present study differed in adult size, relative shell growth, mating propensity and egg size.

In conclusion, the present study showed that soil type can affect reproductive traits in *A. arbustorum*. However, some reproductive traits were also influenced by the origin of the snails and by shell size emphasizing the importance of proper design and replication of life-history studies in gastropods.

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# Chapter II

## 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.

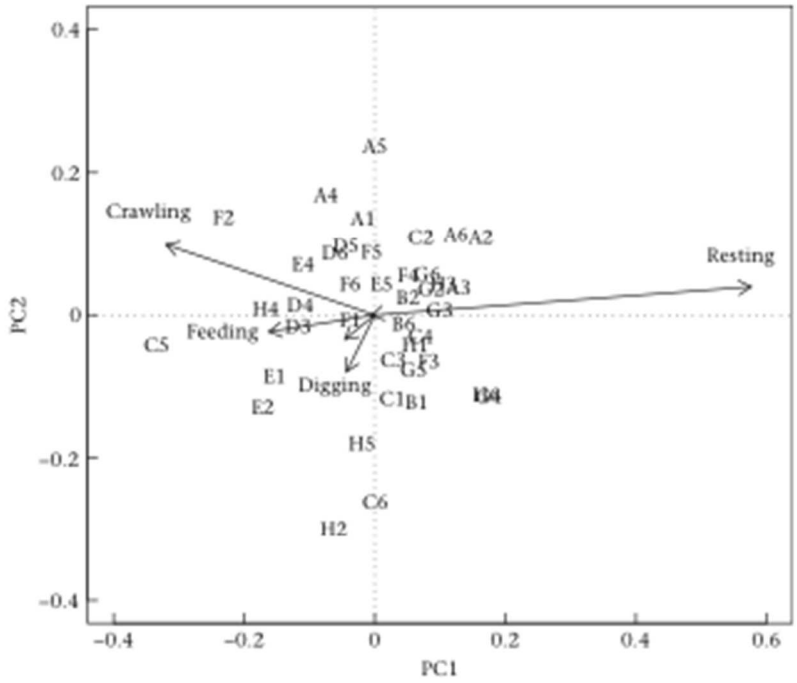


**Figure 1.** Results of the principal components analysis on the behavioural variability of individual snails ($N = 40$). Types of behaviours are represented by arrows and snails are indicated by the observation chamber (letters A–H) and the individual label (1–6) within chamber. For clarity, only crawling,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

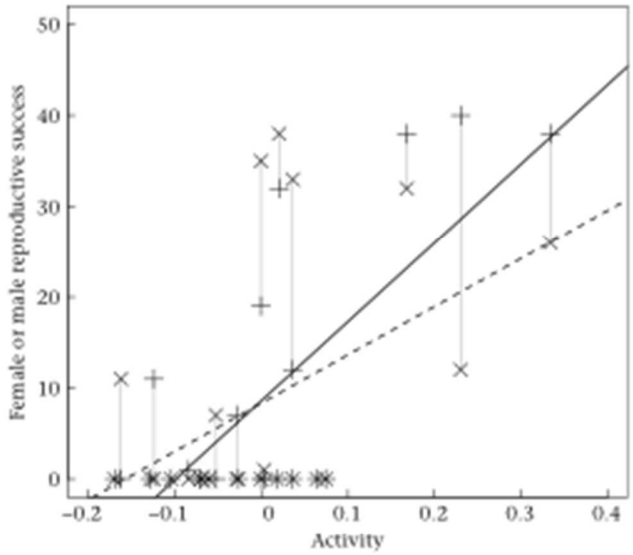


**Figure 2.** Relationships between activity (*A*) and both female and male reproductive success (FRS and MRS) of individual snails: +, continuous line: FRS; x, dashed line: MRS. Data from the four observation chambers with no snail mortality are shown. Regression lines are: FRS = 8.598 + 86.844*A*, $R^2 = 0.54$, $N = 24$, $P < 0.001$; MRS = 8.338 + 53.046*A*, $R^2 = 0.22$, $N = 24$, $P = 0.021$. 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 III

## Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail *Arianta arbustorum*

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ORIGINAL ARTICLE

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# Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail *Arianta arbustorum*

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**Abstract** Sperm competition models on the evolution of sperm size assume associations with another sperm quality trait, sperm longevity. Sperm length can also provide an indication of possible mechanisms affecting motility and thus fertilization success. Despite their importance, however, detailed mechanisms of sperm competition at the gamete level are poorly understood. In simultaneously hermaphroditic land snails, sperm traits and cryptic female choice are assumed to be crucial in determining fertilization success. We examined the variation in sperm length and number among individuals from four natural populations of the land snail *Arianta arbustorum*, a species with multiple mating and long-term sperm storage. We also assessed variation in velocity, motility and longevity of sperm in snails from two of the four populations. Independent of shell size, sperm length differed among populations and, to a minor extent, even among individuals within populations. Mean sperm length of a snail was not correlated with the number of sperm delivered in a spermatophore. The mean sperm velocity (=VCL) did not differ between snails from two populations. However, VCL varied among snails. Percentage motility and longevity of sperm differed between snails from the two populations. No correlations were found between length, velocity, percentage motility and longevity of sperm. To conclude, individual snails differed in sperm quality, and this variation may partly explain the differential fertilization success between *A. arbustorum* snails. Moreover, our findings did not support the positive association between sperm length and longevity assumed by sperm competition models for internally fertilizing species.

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**Keywords** Sperm length · Sperm longevity · Sperm velocity · Sperm number · Sperm competition

## Introduction

Variation in sperm quality, such as sperm size and measures of motility (velocity, percentage motility, and longevity), is widespread among animals and has been observed both between and within species (reviewed in Ward 1998 and Snook 2005). The term "quality" has been used to distinguish these traits from the effect of sperm number on male fertilization success (see Snook 2005). Variation in sperm quality among individuals is relevant for the evolution of sperm characteristics through postcopulatory sexual selection (sperm competition and cryptic female choice). In promiscuous species, sperm function as an integrated unit to ensure both fertility and to maximize the paternity of a male by outcompeting sperm from other males (Birkhead and Møller 1998) or by manipulating female reproduction (Peng et al. 2005). Therefore, phenotypic and/or genetic covariation can be expected between either different measures of sperm quality or sperm quality and sperm number (e.g., Moore et al. 2004; Birkhead et al. 2005).

Investment in sperm quality traits and in sperm number must be considered when examining the evolution of sperm characteristics through sperm competition. Theoretical models predict that the numerical superiority of sperm is an adaptive strategy to increase male fertilization success (Parker 1993; Parker and Begon 1993). Indeed, in several promiscuous species, a common male response to sperm competition is to increase the number of sperm delivered (e.g., Schulte-Hostedde and Millar 2004). Sperm competition models predict that sperm size can confer a fertilization advantage, but only under the assumption of a functional relationship between sperm quality traits. Sperm length might not change or increase or decrease with sperm longevity depending on the mode of reproduction (Parker 1998). Sperm size can increase under conditions of high sperm competition (e.g., the nematode *Caenor-*

*habditis elegans*, LaMunyon and Ward 2002). However, decreased sperm competition did not consistently alter sperm length in flies (Hosken et al. 2001; Pitnick et al. 2001). Studies showed that males producing larger sperm might enhance their fertilization success (e.g., the bulb mite, Radwan 1996; the prosobranch snail *Viviparus ater*, Oppliger et al. 2003). More recently, other relevant ejaculate characteristics such as sperm velocity, motility, and longevity and the interaction among these traits on fertilization success have received increasing attention (e.g., Burness et al. 2004; Gage et al. 2004). Theory for internally fertilizing species predicts that enhanced sperm competition risk would favor increased sperm length when larger sperm enjoy higher survival and could be stored until fertilization (Parker 1998). Though studies so far indicate that sperm longevity decreases with sperm size in externally fertilizing fishes (Stockley et al. 1997; Gage et al. 2002), there is no information available on this relationship for internal fertilizers (Simmons 2001; Snook 2005).

Sperm morphology can provide an indication of possible mechanisms by which motility is affected. Theory predicts that longer sperm generate greater flagellar forces, and swim faster (Katz and Drobnis 1990). However, recent studies suggest that longer sperm are not faster than shorter sperm (Atlantic salmon, Gage et al. 2002; zebra finch, Birkhead et al. 2005). Sperm length and sperm motility are likely both important in determining male fertilization success in internally fertilizing species with sperm storage (e.g., Froman 2003; Snook 2005). Sperm motility predicts fertilization success in competitive situations for many species (e.g., Birkhead et al. 1999; Kupriyanova and Havenhand 2002). Moreover, Rogers and Chase (2002) suggested that in the snail *Helix aspersa*, the beating of longer sperm should generate resistance to incoming sperm in the sperm storage organ.

Sperm characteristics have so far been examined exclusively in gonochoristic species, with the exception of the hermaphroditic nematode *C. elegans* (LaMunyon and Ward 2002). Sexual selection is also likely to shape sperm characteristics in simultaneous hermaphrodites, even though this may conflict with the sperm receiver's interests (Michiels 1998). The sperm donor must persuade the sperm receiver to use its sperm to fertilize eggs, and/or to avoid the postcopulatory control mechanisms (Michiels 1998). In gastropods, the interspecific variation in sperm morphology has been studied and is used as a taxonomical character (e.g., Healy 1996), while the intraspecific variation in sperm traits has not yet been analyzed quantitatively. Spermatozoa of terrestrial gastropods are among the longest within molluscs (e.g., 850 µm in *Helix pomatia*, and 1,140–1,400 µm in *Hedleyella falconeri*; Thompson 1973). The present study focuses on intraspecific variation in sperm characteristics in the simultaneously hermaphroditic land snail *Arianta arbustorum*. In this species, sperm are monomorphic and ca. 800 µm long (Bojat et al. 2001). There is experimental evidence for sperm competition in *A. arbustorum*: multiple mating is common (Baur 1994), viable sperm from different males can be stored for long periods (Baur 1988a), and multiple paternity and differential male fertilization success have been recorded (Baur 1998). *A. arbustorum* does not adjust the number of sperm delivered to the actual risk of sperm competition (Locher and Baur 2000), and its female role has some control over the fertilization of the eggs by selective sperm use (Baur 1994; Bojat and Haase 2002).

Sperm competition is one of the principal determinants of male fitness in species in which females mate promiscuously, but the selective pressures it causes are only partly understood, especially with respect to sperm characteristics favored under sperm competition. To investigate the underlying causes of differential fertilization success between snails, we assessed among- and within-population variation in sperm length and number of sperm transferred during copulation in *A. arbustorum* from four natural populations. To test the assumptions of sperm competition models on the evolution of sperm size (Parker 1998), we measured the velocity, motility, and longevity of sperm, and we assessed their relationship with sperm length in two of the examined populations. We observed that the sperm of *A. arbustorum* leave the spermatophore in bundles to reach the storage organ. Therefore, we also measured the velocity of sperm swimming in bundles. In pulmonate gastropods, sperm bundles have so far been observed in *H. pomatia* (Meisenheimer 1912).

## Materials and methods

### Study organism

*A. arbustorum* is common in moist habitats of northwestern and central Europe (Kerney and Cameron 1979). The snail has determinate growth (adult shell width 16–24 mm; Baur 1984). Individuals become sexually mature at 2–4 years, and adults live another 3–4 years (Baur and Raboud 1988). Mating in *A. arbustorum* includes elaborate courtship behavior, which lasts 2–18 h (Baur and Baur 1992). Copulation is reciprocal; after intromission, each snail transfers simultaneously one spermatophore, which is formed and filled with sperm during copulation (Hofmann 1923; Baminger and Haase 2001). In the field, *A. arbustorum* mates repeatedly in the course of a reproductive season, and fertile sperm can be stored for more than 1 year (Baur 1988a). Snails deposit one to three egg batches consisting of 20–50 eggs (Baur 1990). Outcrossing is the dominant mode of reproduction in *A. arbustorum* (Chen and 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 and Baur 1993). However, the reproductive success of selfing individuals was less than 2% of that of outcrossing snails.

### General method

Virgin snails were obtained by collecting subadult individuals of *A. arbustorum* from four localities in Switzer-

**Table 1** Reproductive traits of *A. arbustorum* from four populations (mean±SE, sample size in parenthesis)

| Variable | Population (year of collection) | | | | Shell size effect[a] | | | Population effect[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Gurnigel (2001, 2002) | Thun (2002) | Nuglar (2001, 2002) | Allschwil (2002) | *df* | *F* | *P* | df | *F* | *P* |
| Shell width (cm)[b] | 1.71(a)±0.01 (58) | 2.04(b)±0.02 (14) | 2.08(b)±0.02 (29) | 2.17(c)±0.02 (17) | – | – | – | 3, 114 | 181.01 | <0.0001 |
| Copulation duration (min) | 421±27 (13) | 388±28 (14) | 432±22 (29) | 423±26 (15) | 1, 66 | 0.08 | 0.78 | 3, 66 | 0.55 | 0.65 |
| Sperm length (μm) | 878(a)±3 (58) | 898(b)±4 (14) | 939(c)±4 (29) | 913(b)±4 (17) | 1, 113 | 0.44 | 0.51 | 3, 113 | 22.59 | <0.0001 |
| Number of sperm delivered ($\times 10^6$) | 2.32(a)±0.13 (57) | 2.52(a)±0.14 (14) | 4.01(b)±0.31 (24) | 5.33(c)±0.57 (16) | 1, 106 | 2.04 | 0.16 | 3, 106 | 7.01 | <0.0001 |
| Spermatophore volume ($mm^3$) | 3.1(a)±0.1 (58) | 2.8(a)±0.4 (14) | 4.3(b)±0.2 (28) | 5.1(b)±0.3 (17) | 1, 112 | 3.15 | 0.08 | 3, 112 | 6.96 | <0.0001 |
| Albumen gland dry weight (mg) | 46.6±4.4 (27) | 58.4±7.1 (14) | 70.2±8.3 (13) | 95.8±11.7 (17) | 1, 66 | 5.29 | 0.03 | 3, 66 | 0.64 | 0.59 |

Differences among populations are indicated by different letters in parentheses (Hochberg's GT2 post hoc test)
[a] *F* and *P* values were calculated from one-way ANCOVAs with shell size as covariate
[b] One-way ANOVA

land. Snails that had not yet completed shell growth were sampled in a coniferous forest near Gurnigelbad (20 km south of Bern; 46°45′N, 7°28′E, 1,230 m a.s.l.), in deciduous forests near Thun (26 km SSE of Bern; 46°44′ N, 7°35′E, at 580 m) and Nuglar (12 km SE of Basel; 47°29′N, 7°42′E, at 430 m), and in an oak–hornbeam forest near Allschwil (4 km SW of Basel; 47°32′N, 7°31′E, at 335 m). Snails from the four localities are, hereafter, referred to as Gurnigel, Thun, Nuglar, and Allschwil populations.

Snails were kept singly in transparent plastic beakers (8 cm deep, 6.5 cm in diameter) lined with moist soil (approximately 4 cm) at 19.5°C with a light–dark cycle of 18:6 h. We cleaned the beakers twice per week and provided fresh lettuce ad libitum as food. Within 4 weeks, subadult individuals reached sexual maturity as indicated by the formation of a flanged lip at the shell aperture.

To examine sperm characteristics, randomly chosen snails (hereafter focal snails) were allowed to copulate with a mating partner (hereafter receiver) from the same population in a transparent plastic container (14×10×7 cm) lined with moist paper towel. The spermatophore produced by the focal snail was obtained by killing the receiver after copulation, and the delivered spermatophore was removed by dissecting the receiver female reproductive tract. We measured the shell width to the nearest 0.1 mm using a vernier calliper to examine possible effects of the size of the focal snail on sperm traits.

We counted the number of sperm and measured their length in snails from all four populations of *A. arbustorum* collected in summer 2001 and 2002 (samples size in Table 1). In each spermatophore, we measured the length ($L$) of the sperm-containing part and its diameter at both ends ($D_1$ and $D_2$) to the nearest 0.1 mm. Spermatophore volume was approximated assuming a truncated-cone volume $\left[V = 1/12\pi L\left(D_1^2 + D_1D_2 + D_2^2\right)\right]$. We also examined possible relationships between the male reproductive output (sperm length or sperm number) on the female reproductive allocation (estimated by albumen gland dry weight) in this simultaneous hermaphrodite. The albumen gland was dissected out of the female reproductive tract of the focal snail. We determined the dry weight of the albumen gland to the nearest 0.1 mg. We also examined the relationships between sperm number, spermatophore volume (cubic millimeters), and copulation duration (minutes).

We measured sperm length and their motility in snails from two (Gurnigel and Nuglar) of the four populations collected in summer 2004 (samples size in Table 3). Snails of these populations showed large differences in sperm length (see Table 1).

## Sperm length

Sperm characteristics were examined in spermatozoa from spermatophores, which were dissected out of the receiver approximately 3 h after copulation. Sperm of *A. arbustorum* are densely packed in the spermatophore (Baminger

and Haase 2001). We developed a technique to isolate a single sperm from the spermatophore without damaging their 800- to 950-µm long tails. Using insect needles, we carefully broke off the wall of the sperm-containing part along the longitudinal axis of the spermatophore. To activate sperm, we placed the spermatophore for 12 h in 500 µl Ringer solution (3.5 g NaCl, 0.05 g KCl, 0.20 g $NaHCO_3$, 0.10 g $CaCl_2$ in 1,000 ml; pH 8.2; cf. Romeis 1989). The suspension with active sperm was used to measure the length of single sperm. For this purpose, aliquots of 16-µl sperm suspension were placed on three microscopic slides. The samples were covered with a coverslip and air-dried. The remaining sperm suspension was concentrated (at 30°C for 60 min in an Eppendorf 5301 concentrator) and added to the spermatophore sample, which was preserved at 4°C for sperm counting (see below).

We digitized randomly chosen sperm using a camera (SONY CCD-Iris) mounted on a compound microscope (Leica DMLD, magnification 200×) connected to a Macintosh computer. From these images, we measured the total sperm length (head and tail) of 25–29 sperm from each spermatophore using the public domain NIH-Image software (version 1.63; http://rsb.info.nih.gov/nih-image).

We assessed the reliability of multiple length measurements ($n$=6) on the same sperm ($n$=9) by calculating the repeatability following Lessells and Boag (1987). Repeatability of sperm length measurements was 0.92, indicating the technique was accurate. Broken tails of sperm could bias our measurements of sperm length. We found ten outliers (i.e., 0.53% of a total of 1,875 sperm measured from 72 snails; Grubbs' test, public domain http://www.graphpad.com/articles/grubbs.htm). However, mean sperm length including these outliers did not differ from mean values in which outliers were excluded (ANOVA: $F_{2,68}$=0.32, $P$=0.58).

## Sperm number

The procedure for sperm counting is described in detail in Locher and Baur (1997). Briefly, the sperm suspension obtained from the mechanically disrupted spermatophore was stained with a gallocyanin–chromium complex (a DNA marker). Two subsamples of known volume of the sperm suspension were transferred to a Bürk–Türk counting chamber. We counted all sperm heads in randomly chosen cells until the total number of sperm heads exceeded 400, and used the average of the two subsamples to calculate the total number of sperm transferred in a spermatophore.

To assess the proportion of sperm removed for sperm length measurements, we counted the number of sperm of three subsamples from each six spermatophores. A total of 1,542–3,271 (mean 2,352.1±264.3) sperm was removed for sperm length measurements. This corresponds to 0.045–0.096% (mean 0.068%) of the total number of sperm in a spermatophore. Consequently, we corrected our estimate of sperm number by multiplying the value with a correction factor of 1.00068.

## Sperm motility: velocity, percentage motility, longevity

Sperm characteristics were examined in sperm from spermatophores, which were dissected from the receiver 1.5 h after copulation. Single spermatophores were placed into the aperture of a 20-µm deep standard microscopic chamber (CASA 2x, Leja, NL) filled with 10 µl Ringer solution. Motile sperm were video-recorded at room temperature (23.7±0.2°C; $n$=8 days) for 10 min using a differential interference contrast microscope (Polyvar, Reichert-Jung, Vienna, Austria; magnification 100×; with the neutral filter N3 and a light transmission of 12.5% to enhance image contrast). The microscopic chamber was checked top-down to minimize the risk of sampling a sperm more than once. "Edge effects" may increase sperm velocity, but this systematic error was the same for all measurements. The spermatophore with the remaining sperm was preserved in 200 µl Ringer solution, and kept at room temperature to assess percentage motility (see below).

Sperm velocity was examined using a computer assisted IVOS-semen analysis system (CASA version 12.1, Hamilton Thorne Research, University Hospital Basel). CASA uses a nearest-neighbor algorithm to select the closest motile object. We adjusted the procedure to *A. arbustorum* sperm by taking 50 frames per second, setting cell detection to a minimum contrast of 50 and minimum cell size to 3 pixels. We measured the average velocity (micrometers per second) of single sperm considering both the actual track velocity (VCL, average velocity, which includes deviations of sperm head movement) and the straight-line velocity (VSL, average velocity measured in a straight line). Sperm velocity (VCL and VSL) was recorded twice (90 and 120 min after sperm activation, i.e., 180 and 210 min after copulation) in 10–12 sperm from each spermatophore.

Velocity of sperm leaving the spermatophore in bundles was measured manually because sperm bundles could not be automatically identified by CASA. We measured VSL of 4–12 sperm bundles twice: when they were leaving the spermatophore and after 10 min in a microscopic chamber. In each case, five successive frames were taken at an interval of 1 s (using the software BioPictViewer+; Zschokke 2003). In four spermatophores, VSL of sperm bundles leaving the spermatophore (82.0±16.7 µm/s) was not significantly different from the VSL of those that moved freely in the microscopic chamber (50.0±9.9 µm/s) coming from the same spermatophore (paired $t$ test: $t$=1.95, $df$=3, $P$=0.15). Some of the sperm bundles disentangled after leaving the spermatophore. This allowed us to compare VSL of sperm in bundles and VSL of singly swimming sperm of the same spermatophore.

We assessed percentage motility (number of active sperm out of 100 randomly chosen sperm) at 3 h intervals beginning 12 h after sperm activation. We determined percentage motility in 10-µl sperm suspension using a light microscope (Leica DMLD, magnification 100×). Repeated

measurements of percentage motility also allow an estimate of sperm longevity, which is defined as time elapsed since activation until 95% of the sperm are inactive.

### Data analysis

Statistical analyses were performed using SPSS 11.0.2 for Macintosh. Differences in coefficient of variation (CV, corrected for different sample sizes) were examined using Levene's test. Differences in reproductive traits (log-transformed data) were examined using one-way analysis of covariance (ANCOVA) with population as fixed factor and shell width as covariate. Differences in percentage motility were examined using one-way repeated measures analysis of covariance. In all cases, the nonsignificant interaction was dropped from the model (Scheiner and Gurevitch 1993). Differences between populations were investigated using the Hochberg's GT2 post hoc test for unequal sample sizes. Variance components for sperm length were partitioned among sources (i.e., among populations, among snails within population, and among sperm within snails) by using nested analysis of variance. Relationships between sperm traits were analyzed with the Pearson correlation test (for small sample sizes).

## Results

### Sperm length

*Among-population variation* Snails differed in mean shell width (Table 1; Fig. 1). However, independent of shell width, mean sperm length of *A. arbustorum* differed among populations (Table 1; Fig. 1). Sperm length ranged from 878±3 µm in the Gurnigel population to 939±4 µm in the Nuglar population. The difference in mean sperm length in the four populations examined accounted for 6.7% of the average sperm length (grand mean: 904 µm).

*Within-population variation* Different individuals produced sperm of different length (one-way ANCOVA: Gurnigel: $F_{57,1429}$=18.34, $P$<0.0001; Thun: $F_{13,356}$=7.67, $P$<0.0001; Nuglar: $F_{28,692}$=15.77, $P$<0.0001; Allschwil: $F_{16,439}$=15.00, $P$<0.0001; Fig. 1). Sperm length was not correlated with shell width (in all populations $P$≥0.08; Fig. 1). The interindividual variation in mean sperm length (CV) was similar for all populations: Gurnigel 2.5% ($n$=58), Thun 3.1% ($n$=14), Nuglar 2.5% ($n$=29), and Allschwil 2.3% ($n$=17) (Levene's test: $df_1$=3, $df_2$=114, $P$=0.31). Mean sperm length of individuals fitted a normal distribution in each population (Shapiro–Wilk test: in all cases $P$≥0.62). Similarly, at the individual level, sperm length was normally distributed in all 118 snails examined, indicating that sperm length is unimodal in *A. arbustorum*. Forty-nine percent of the variation in sperm length can be attributed to differences among populations, 17.3% to differences among snails within population, and 33.7% to differences within individuals (Table 2). Thus, snails

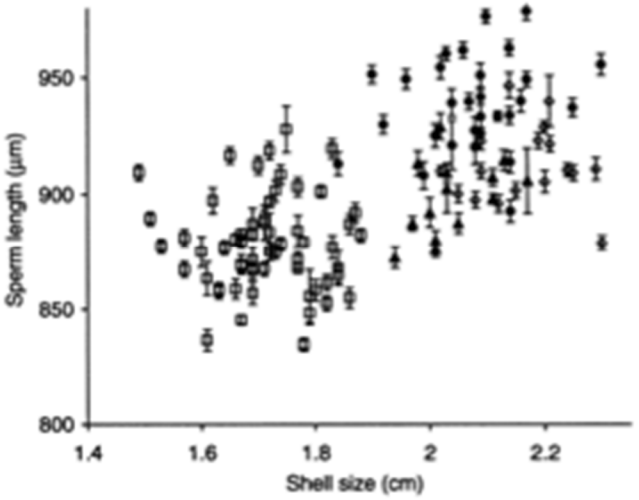


**Fig. 1** Sperm length (mean±SE) of *A. arbustorum* from four populations [*open square*, Gurnigel (2001, 2002), $n$=58 snails; *filled triangle*, Thun (2002), $n$=14; *filled circle*, Nuglar (2001, 2002), $n$=29; *open diamond*, Allschwil (2002), $n$=17]. Twenty-five to twenty-nine sperm were measured from each snail

differed in sperm length both among and—to a minor extent—within populations.

*Among-year variation* Snails from different populations produced sperm of different length; this difference was consistent among years (one-way ANCOVA: shell size $F_{1,82}$=0.11, $P$=0.74; year: $F_{1,1.06}$=1.46, $P$=0.43; population: $F_{1,2.90}$=20.01, $P$=0.02; year×population: $F_{2,82}$=5.60, $P$=0.02). The significant interaction term indicates that not all populations showed the same pattern. In fact, in the Nuglar population, sperm length differed between years (two-way ANCOVA: shell size $F_{1,26}$=0.01, $P$=0.93; year: $F_{1,26}$=11.80, $P$<0.002), probably due to a sampling effect (data from 2001: $n$=16 snails; from 2002: $n$=13 snails).

### Sperm number

*Among-population variation* Independent of shell width, the snails from the four populations transferred different numbers of sperm in their spermatophores (Table 1;

**Table 2** Results of the nested analysis of variance for sperm length (four populations; 14–58 snails per population; 25–29 sperm per snail)

| Source of variation | *df* | *MS* | *F* | *P* | Percentage of total |
|---|---|---|---|---|---|
| Populations | 3 | 617,226.23 | 992.58 | <0.0001 | 49.0 |
| Snails within populations | 114 | 9,936.72 | 15.98 | <0.0001 | 17.3 |
| Sperm within snails | 2,916 | 621.84 | | | 33.7 |
| Total | 3,034 | 1,843.66 | | | 100 |

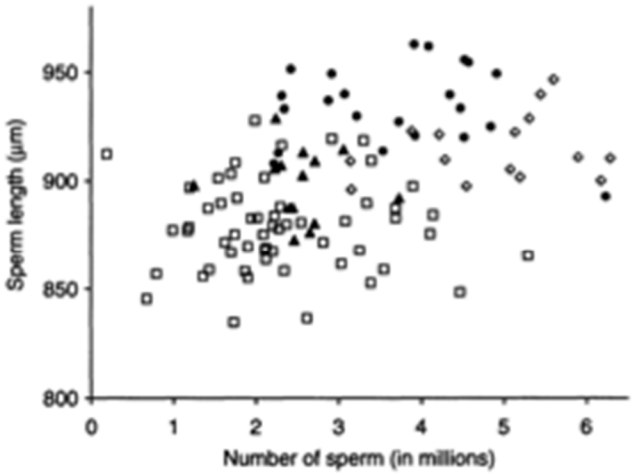


**Fig. 2** Relationship between sperm length and number of sperm transferred during copulation in *A. arbustorum* from four populations [*open square*, Gurnigel (2001, 2002), $n$=57 snails; *filled triangle*, Thun (2002), $n$=14; *filled circle*, Nuglar (2001, 2002), $n$=24; *open diamond* Allschwil (2002), $n$=16)]

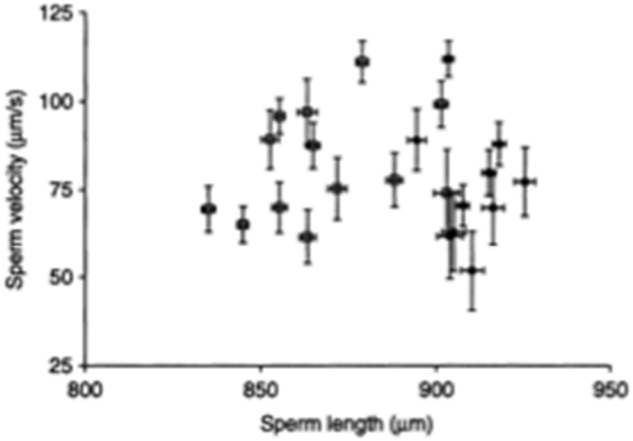


**Fig. 3** Relationship between sperm velocity (VCL) and sperm length in *A. arbustorum* from two populations (data from 2004: *open square*, Gurnigel, $n$=13; *filled circle*, Nuglar, $n$=10). For each snail, VCL of 10–12 sperm and the length of 25–29 sperm were measured (values are means±SE)

Fig. 2). The mean number of sperm delivered ranged from $2.3\times10^6$ in the Gurnigel population to $5.3\times10^6$ in the Allschwil population (Table 1). Populations differed also in spermatophore volume, but not in copulation duration (Table 1). Spermatophore volume was positively correlated with sperm number (pooled data after removing the effect of shell size: $r$=0.34, $n$=111, $P$<0.0001). As observed in previous studies for *A. arbustorum* (e.g., Locher and Baur 1999), sperm number was not influenced by copulation duration (pooled data after removing the effect of shell size: $r$=0.18, $n$=65, $P$=0.15).

*Within-population variation* The number of sperm transferred during copulation did not correlate with shell width (in all populations $P\geq0.57$). The interindividual variation in sperm number within the populations examined (expressed as CV) was high: Gurnigel 43.6% ($n$=57), Thun 21.5% ($n$=14), Nuglar 37.7% ($n$=24), and Allschwil 38.3% ($n$=16).

## Relationship between sperm length and number and female reproductive allocation

Considering all snails from the four populations, mean sperm length was positively correlated with the number of sperm transferred during copulation (log-transformed data: $r$=0.43, $n$=111, $P$<0.001; Fig. 2). However, this correlation was no longer significant after removing the effect of shell size (residuals from sperm length–shell width and sperm number–shell width relationships: $r$=0.02, $n$=111, $P$=0.81). Similarly, considering snails from each population separately, mean sperm length was not correlated with the number of sperm delivered (in all populations $P\geq0.10$; Fig. 2). Thus, sperm length and the number of sperm transferred during copulation are independent reproductive traits in *A. arbustorum*.

In simultaneous hermaphrodites, reproductive allocation to the male function (i.e., sperm production) is not independent on reproductive allocation to the female function (egg production). We used albumen gland dry weight as an estimate for female reproductive allocation (Table 1). However, neither sperm length nor sperm number were correlated with albumen gland weight (pooled data after removing the effect of shell size: albumen gland weight–sperm length: $r$=0.11, $n$=71, $P$>0.35; albumen gland weight–sperm number: $r$=0.03, $n$=70, $P$>0.83).

## Sperm motility

*Variation in sperm velocity* VCL, the mean velocity of the actual sperm track, was recorded twice for each spermatophore at 120 and 190 min after activation; mean VCL of the two samples did not differ (paired $t$ test: $t$=1.03, $df$=17, $P$=0.32). Independent of shell width, mean VCL did not differ between snails from the Gurnigel and Nuglar populations (one-way ANCOVA: shell width: $F_{1,20}$=0.01, $P$=0.92; population: $F_{1,20}$=0.42, $P$=0.52; Fig. 3). Thus, for further analyses we pooled the data on sperm velocity from snails of both populations.

Mean VCL (79.8±3.3 µm/s, $n$=23) differed among individual snails (one-way ANOVA: $F_{22,333}$=5.70, $P$<0.001). VCL ranged from 52 to 112 µm/s and showed an interindividual variation (expressed as CV) of 33% ($n$=23; Fig. 3). The distribution of mean VCL values did not differ from a normal distribution (Shapiro–Wilk test: $df$=23, $P$=0.47; Fig. 3). Furthermore, VCL values of all sperm measured in each spermatophore fitted a normal distribution (Shapiro–Wilk test: for all 23 snails $P\geq0.08$). Similar results were obtained when sperm velocity was measured as straight-line velocity (VSL: 20.2±1.1 µm/s, $n$=23; data not shown).

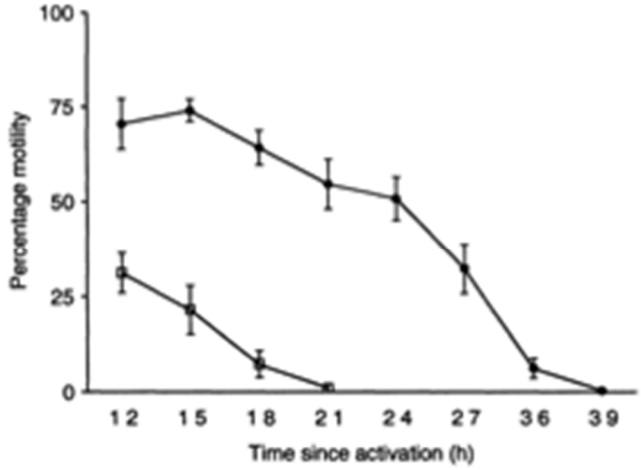


**Fig. 4** Mean percentage motility as a function of time in *A. arbustorum* from two populations (data from 2004: *open square*, Gurnigel, $n$=6–7; *filled circle* Nuglar, $n$=5–11)

*Velocity of sperm in bundles* Sperm were observed to leave the spermatophore in bundles, which consisted of 50–100 sperm oriented in the same direction with wrapped flagella that beat synchronously. Mean VSL was calculated from 4–12 bundles per spermatophore. In the microscopic chamber, VSL of sperm bundles (53.0± 5.1 µm/s, $n$=9) did not differ from VSL of singly swimming sperm (54.8±4.2 µm/s, $n$=9; paired $t$ test: $t$=0.29, $df$=8, $P$=0.78).

*Percentage motility and longevity* Percentage motility (the number of active sperm out of 100 sperm observed) was not affected by shell width; populations differed in percentage motility (one-way repeated measures ANCOVA: shell width: $F_{1,14}$=3.82, $P$=0.07; population: $F_{1,14}$=34.24, $P$<0.0001; time: $F_{2,28}$=2.63, $P$=0.09; Fig. 4). No sperm were motile after 21 h in snails from the Gurnigel population and after 39 h in snails from the Nuglar population (Fig. 4). Sperm longevity (time elapsed since activation until 95% of the sperm were inactive) differed between populations (Gurnigel 18.9±1.5 h, $n$=7; Nuglar 35.5±1.2 h, $n$=9), but was not affected by shell width (one-way ANCOVA: shell width: $F_{1,13}$=0.01, $P$=0.93; population: $F_{1,13}$=53.6, P<0.0001).

Relationships between sperm length and motility

No correlations between sperm length and motility parameters were found within populations (Table 3). Sperm length and sperm velocity were not correlated in pooled data from both populations (residuals from sperm length–shell width and sperm number–shell width relationships: $r$=0.21, $n$=23, $P$=0.34). Sperm in the Nuglar population were, on average, 61 µm longer than sperm in the Gurnigel population (Table 1) but they did not swim faster (Fig. 3). Furthermore, no correlations were found between sperm motility parameters (for all comparisons: Gurnigel $P$≥0.19; Nuglar $P$≥0.19). Thus, the sperm quality traits examined varied independently.

**Table 3** Correlations between mean sperm length and sperm motility traits in snails from two *A. arbustorum* populations (data from 2004)

| | Gurnigel | | | Nuglar | | |
|---|---|---|---|---|---|---|
| | $n$ | $r$ | $P$ | $n$ | $r$ | $P$ |
| VCL (µm/s) | 13 | 0.39 | 0.19 | 10 | −0.14 | 0.70 |
| Percentage motility | | | | | | |
| 12 h after activation | 7 | 0.04 | 0.94 | 5 | 0.80 | 0.10 |
| 18 h after activation | 7 | −0.04 | 0.94 | 11 | 0.51 | 0.11 |
| Sperm longevity (h) | 7 | −0.10 | 0.83 | 9 | 0.20 | 0.61 |

## Discussion

The present study showed significant differences in sperm length, both among- and within-populations of *A. arbustorum*. To our knowledge, we present for the first time quantitative evidence for intraspecific sperm length variation in a simultaneously hermaphroditic gastropod. Different processes of postcopulatory sexual selection may result in sperm size differences (sperm competition, e.g., LaMunyon and Ward 2002; cryptic female choice, e.g., Pitnick et al. 2003), which in turn may lead to different paternity success (e.g., Oppliger et al. 2003). Therefore, interindividual differences in sperm length could account for the unexplained variance in fertilization success in *A. arbustorum* (Baur 1998).

Intraspecific variation in sperm length is widespread in gonochoristic animals (reviewed in Ward 1998 and Snook 2005). However, most of the previous studies focused on differences in sperm length among individuals. We found also considerable differences in sperm length among *A. arbustorum* populations, which could not be explained simply by differences in snail size. This is interesting, as most reproductive traits (e.g., egg size, clutch size) are size-related in *A. arbustorum* (Baur 1988b, 1990; Baur and Raboud 1988; Baur et al. 1998). The difference in sperm length observed between populations could be a result of (1) genetic differences between populations, (2) a bias introduced by sampling, and/or (3) different phenotypically plastic responses by animals sampled under different field conditions. These explanations are not mutually exclusive. Parent–offspring and sib comparisons revealed a significant heritability of sperm length in *A. arbustorum*, indicating a genetic basis (Minoretti et al., in preparation). Sample size effects might be small, as we examined sperm length in 58, 29, 16, and 14 individuals from the four populations. Sperm length was also significantly different between the two populations with the largest sample sizes (Gurnigel: $n$=58; Nuglar: $n$=29). In these two populations, sperm length was measured in 2 years (2001 and 2002). Sperm length did not differ among years in the Gurnigel population with relatively large sample sizes (30 and 28 snails in the 2 years). In the Nuglar population, however,

sperm length differed between snails examined in 2001 and 2002. This difference could be due to relatively small sample sizes (16 and 13 snails).

We measured total sperm length because the borders between head, midpiece, and tail are indistinct in helicid spermatozoa (Dohmen 1983). The sperm head of *A. arbustorum* measures approximately 8 µm (1% of the total sperm length; Bojat et al. 2001). It is therefore unlikely that sperm head size accounted for the interindividual differences in sperm length. Thus, *A. arbustorum* produces monomorphic spermatozoa (Bojat et al. 2001), which vary in length. Individuals of *A. arbustorum* showed consistent sperm length in two successive matings (Minoretti et al., in preparation). Furthermore, sperm length was not affected by snail size and the weight of the albumen gland (a measure for female reproductive allocation). This suggests that sperm length in *A. arbustorum* is not dependent on body conditions, confirming similar studies on gonochoristic animals (e.g., Hellriegel and Blanckenhorn 2002; Schulte-Hostedde and Millar 2004). It remains to be determined whether snails with long sperm actually enjoy an increased fertilization success in multiply mated snails.

An intriguing aspect related to variation in sperm characteristics is that for quantitative traits like sperm size and sperm velocity, different "groups" of sperm within ejaculates may exist (e.g., Thurston et al. 1999, 2001). Although differences in sperm length are continuous within individuals of *A. arbustorum*, our results indicate that sperm length within the same snail is highly variable (the within-individual variation explained 33.7% of total sperm length). The presence of specific "groups" within ejaculates may provide a selective fertility advantage (e.g., Thurston et al. 1999, Baer et al. 2003). Bet-hedging for variable sperm may be selected for when it allows either differential ejaculation of a certain length fraction after recognition of the type of partner, or differential storage of a certain length fraction by partners of specific types (Baer et al. 2003). At least in some sperm-dimorphic species, females seem to be able to discriminate between groups of morphologically distinct sperm. For example, only short sperm were found in the spermatheca of *Drosophila subobscura* (Bressac and Hauschteck-Jungen 1996) and the cicada *Graptopsaltria nigrofuscata* (Kubo-Irie et al. 2003). In contrast, only long sperm enter the external layer of eggs in six *Drosophila* species (Karr and Pitnick 1996). In *A. arbustorum*, the female role may have some control over the fertilization of the eggs by selective sperm use (Baur 1994; Bojat and Haase 2002). Therefore, sperm length variation could also result from selective sperm storage by females (e.g., Miller and Pitnick 2002).

Independent of snail size differences, *A. arbustorum* individuals transferred different amounts of sperm during copulation. Sperm number was correlated neither with shell width nor with albumen gland weight. In *A. arbustorum*, sperm production seems to be less constrained by energy and nutrient uptake than egg production (Locher and Baur 2002). When sperm competition is fundamentally analogous to a raffle, the relative number of sperm will be the primary determinant of fertilization success (Parker 1993; Parker and Begon 1993). However, *A. arbustorum* does not adjust the number of sperm delivered to the actual risk of sperm competition (Locher and Baur 2000). In our study, sperm length did not trade-off with sperm number; as predicted by theory, these traits should vary independently under sperm competition (Parker 1998). The smaller coefficient of variation of sperm length compared to sperm number could indicate that sperm size is under stronger selection. Thus, sperm length may be a better predictor of fertilization success than the number of sperm transferred during copulation in *A. arbustorum*.

Interindividual differences in sperm motility could result in differential fertilization success (sperm velocity, e.g., Kupriyanova and Havenhand 2002; percentage motility, e.g., Froman et al. 2002). Snails differed in sperm motility parameters; the effect of sperm motility on fertilization success has not yet been examined in *A. arbustorum*. After copulation, sperm leaving the spermatophore should avoid the bursa copulatrix, where they are immobilized rapidly and digested (Lind 1973); only 0.1% of the sperm delivered reach the storage organ (spermatheca). Thus, interindividual differences in sperm velocity and percentage motility could enhance snail fertilization success by increasing the number of sperm that escape the bursa copulatrix and reach the spermatheca. Sperm swimming in bundles did not swim faster than singly swimming sperm, but sperm bundles could likely generate higher trusting forces and move along the female tract (e.g., Hayashi 1998). Thus, the transfer of sperm in bundles could be also an adaptation to overcome the "sperm trap" bursa copulatrix. Furthermore, there is some evidence that sperm motility may influence sperm storage (e.g., Rogers and Chase 2002; Froman 2003).

Longer sperm may be advantageous under conditions of sperm competition if they swim faster than shorter sperm as predicted by theory (Katz and Drobnis 1990). There is empirical evidence supporting this fact from species comparisons (mammals, Gomendio and Roldan 1991; *Drosophila* sp., Joly et al. 1991). However, there is no evidence of a relationship between sperm size and velocity within species, for either external (Atlantic salmon, Gage et al. 2002) or internal (zebra finch, Birkhead et al. 2005) fertilizers. We did not find any direct association between sperm length and sperm velocity in *A. arbustorum*, confirming these previous studies. Although snails from the two populations examined differed in mean sperm length, the swimming velocity of sperm did not differ. Sperm length could also contribute to *A. arbustorum* fertilization success if longer sperm generate greater flagellar forces as predicted by theory (Katz and Drobnis 1990). Sperm thrusting force is likely to be an important trait for gamete competence in internal fertilizers, increasing the probability of the sperm reaching the storage organ.

In species with internal fertilization and long-term sperm storage, theory predicts that sperm length can confer a fertilization advantage provided there is a positive association between sperm length and longevity (Parker 1998). So far, no study has examined this relationship in simul-

taneous hermaphrodites, nor in other internally fertilizing species with long-term sperm storage (see Snook 2005). In the Atlantic salmon, sperm length was not correlated with sperm velocity, but sperm length and longevity were negatively associated (Gage et al. 2002). In *A. arbustorum*, viable sperm could be stored in the spermatheca up to 1 year after copulation (Baur 1988a). There is no indication that sperm are nourished through the epithelium of the female reproductive tract in *A. arbustorum* (Bojat et al. 2001). In helicid snails, the spermatozoa are richly supplied with glycogen granules as an energy supply for sperm motility (Anderson and Personne 1976). In *A. arbustorum*, the glycogen granules are arranged in a helical structure along the axoneme of the sperm tail to the end of the flagellum (Bojat et al. 2001). Thus, longer flagella might contain more resources, resulting in large sperm with a higher energy supply for motility and/or longevity than smaller ones. Indeed, the longer sperm of snails from the Nuglar population survived longer than the shorter sperm of snails from the Gurnigel population. Within populations, however, no relationship between sperm length and longevity was found. Moreover, sperm velocity was not correlated with sperm longevity. Further work is needed to assess the energy supply of *A. arbustorum* sperm (ATP and/or glycogen content). For example, in domestic fowl, longer sperm have increased motility because of their higher ATP/mitochondria content (Froman et al. 1999, 2002).

Our data did not reveal any association between sperm size (total length) and sperm function (velocity, motility and longevity). Our findings do not support the assumptions of sperm competition models on the evolution of sperm size for internally fertilizing species like *A. arbustorum* (Parker 1998). Several of these comparisons could be nonsignificant due to low power in the statistical tests. To reach a power of 80% ($\alpha$=0.05), sample sizes of >190 snails are required. However, the use of phenotypic measures to assign relationships between ejaculate traits might not be conclusive (Moore et al. 2004; Snook 2005). Future research should examine genetic correlations between sperm traits to elucidate the constraints on the evolution of sperm morphology and function (e.g., Moore et al. 2004).

Our results are relevant for understanding the mechanisms underlying fertilization success in simultaneous hermaphrodites. We found interindividual differences in sperm quality that can explain the differential paternity success of *A. arbustorum* individuals (Baur 1998). Considerable variation was also detected within individuals and among populations of *A. arbustorum*. Thus, the intraspecific variation in sperm traits in nature is likely to be even greater than reported here and in similar studies (reviewed in Ward 1998 and Snook 2005). Models of sexual selection for the evolution of sperm morphology and function should take into account different processes of postcopulatory sexual selection (e.g., Snook 2005). In this study, we showed how the interindividual differences in the sperm quality traits found in *A. arbustorum* could be a response to sperm competition risk in interaction with cryptic female choice.

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# Chapter IV

## Heritability of sperm length and adult shell size in the land snail *Arianta arbustorum* (Linnaeus, 1758)

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# HERITABILITY OF SPERM LENGTH AND ADULT SHELL SIZE IN THE LAND SNAIL *ARIANTA ARBUSTORUM* (LINNAEUS, 1758)

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## ABSTRACT

Sperm length varies considerably, both between and within species, but the evolutionary implications of this variation are poorly understood. Sexual selection on sperm length requires a significant additive genetic variance, but few studies have actually measured this. Stylommatophoran gastropods have extraordinarily long sperm. However, the extent of intraspecific variation has rarely been examined. Here we present the first estimates of heritability of sperm length in the land snail *Arianta arbustorum* using two complementary approaches (one-parent–offspring regression and full-sibling split design). We also examined whether sperm length is influenced by the shell size of the snail and estimated heritability of shell size. Sperm delivered by the same individuals in 2–4 matings over two reproductive seasons did not differ in length, indicating a high repeatability of this trait. Offspring of 10 families were kept at three temperatures (11, 15 and 20°C) to examine the influence of different environmental conditions on sperm length and adult shell breadth. Independent of shell breadth, sperm length was affected by temperature but not by family of origin (the variance component associated with family was not significantly different from zero), while adult shell breadth was influenced by temperature and family of origin. Higher temperatures resulted in shorter sperm, but larger shells. The heritability of sperm length derived from the two different approaches (one-parent–offspring regression: $h^2 \pm \mathrm{SE} = 0.52 \pm 0.55$; full-sibling split design: $H^2 \pm \mathrm{SE} = -0.19 \pm 0.28$) suggests relatively little genetic variation in this trait in the studied population. In contrast, the heritability of adult shell breadth indicates a strong genetic effect (mother–offspring regression, $h^2 \pm \mathrm{SE} = 0.90 \pm 0.33$). The heritability ($h^2 \pm \mathrm{SE}$) of adult shell breadth obtained from the father–offspring regression was $0.18 \pm 0.42$, i.e. five times smaller than that of the mother–offspring regression, suggesting a maternal effect on shell size.

## INTRODUCTION

Sperm length shows an extraordinary variation both within and among species (Snook, 2005; Pitnick, Hosken & Birkhead, 2009a). This variation may reflect population- and species-specific differences in fertilization mode, allometry and strength of postcopulatory sexual selection. Within species, sperm–female interactions have been shown to be a major factor influencing sperm length evolution (e.g. Miller & Pitnick, 2002; Pattarini *et al.*, 2006; Pitnick, Wolfner & Suarez, 2009b). The length of sperm may influence their power and swimming speed as well as longevity because of differences in the energetic demands between longer and shorter flagella (e.g. Mossman *et al.*, 2009; Helfenstein, Podevin & Richner, 2010). In taxa with sperm storage organs, sperm length may determine the ability to reach the storage organs first and to move to the ovum from the storage organ once ovulation takes place (e.g. in the land snail *Helix aspersa*, Roger & Chase, 2002; in the domestic fowl, Froman, 2003).

The influence of sperm competition on sperm length is less well understood (Pizzari & Parker, 2009). In some species, sperm length appears to be relevant for male reproductive success under conditions of intensive sperm competition. For example, short sperm were favoured during sperm competition in the cricket *Gryllus bimaculatus* (Gage & Morrow, 2003) and sperm morphometry was adjusted in males of the polymorphic Gouldian finch (*Erythrura gouldiae*) across social environments (Immler, Calhim & Birkhead, 2010). Similarly, studies on simultaneous hermaphrodites indicate that the intensity of sperm competition can affect sperm length (Crean & Marshall, 2008; but see Janicke and Schärer 2010). In contrast, no correlation between sperm length and male reproductive success could be found in a variety of species including insects (Tomkins & Simmons, 2000; Simmons *et al.*, 2003), fish (Gage *et al.*, 2004) and mammals (Gage & Freckleton, 2003).

Sperm length usually exhibits little variation across ejaculates of single males (Morrow & Gage, 2001a; Birkhead *et al.*,

2005; Immler *et al.*, 2008; Fitzpatrick & Baer, 2011), indicating strong genetic determination (Morrow & Gage, 2001b; Simmons & Kotiaho, 2002). Simmons & Moore (2009) summarized available information on additive genetic and phenotypic variation and heritabilities of sperm and sperm-related traits in various taxa. Heritabilities of sperm morphology including sperm length varied around 0.5. In contrast, heritabilities of sperm performance traits such as sperm motility, viability and fertilization success were lower. Indeed, fitness traits are considered to show a substantial genetic variation, but low heritabilities due to a large fraction of residual variation (Houle, 1992). However, there is some evidence that environmental factors may influence sperm length. Sperm size increased with temperature in dung flies (Blanckenhorn & Hellriegel, 2002; but see Gage & Cook, 1994), and with the males' age in the rove beetle *Alloeocharo bilineata* (Green, 2003), but decreased with larval density in *Drosophila melanogaster* (Morrow, Leijon & Meerupati, 2008; but see Gay *et al.*, 2009). The type of nutrition showed only a weak effect on sperm size (Gage & Cook, 1994; Amitin & Pitnick, 2007). Furthermore, maternal effects may partly determine sperm length. In the seed beetle *Callosobruchus maculates*, older mothers produced sons with longer sperm than did younger mothers (Dowling, Nowostawski & Arnqvist, 2007; Gay *et al.*, 2009). However, most aspects of the control of sperm length, including physiological processes and temperature, have so far not been investigated (Engel, Ludington & Marshall, 2009).

Gastropods exhibit a large interspecific variation in sperm morphology (Thompson, 1973; Healy, 1988, 1996; Luchtel *et al.*, 1997). Spermatozoa of stylommatophorans are among the largest of the gastropods (e.g. 800 μm in *Arianta arbustorum*, Bojat, Sauder & Haase, 2001; 850 μm in *Helix pomatia* and 1140–1400 μm in *Hollyella falconeri*, Thompson, 1973). Information on intraspecific variation in sperm length is restricted to a single species, the land snail *Arianta arbustorum* (Linnaeus, 1758) (Minoretti & Baur, 2006). However, the significance of the variation in sperm length in terrestrial gastropods is largely unknown.

In the present study, we assessed the repeatability of sperm length in spermatophores delivered in successive matings by individuals of *A. arbustorum*. We also conducted a breeding experiment to estimate the heritability of sperm length and adult shell breadth in this species. We considered the relationship between shell breadth and sperm length because most reproductive traits (e.g. egg size, clutch size) are size-related in *A. arbustorum* (Baur, 1988b, 1990; Baur & Raboud, 1988; Baur, Locher & Baur, 1998). We used two different methods to estimate the heritability of sperm length and shell breadth. Firstly, we calculated the one-parent–offspring regression to obtain an estimate of narrow-sense heritability $h^2$. Secondly, we used a full-sibling split design to raise the offspring of several snails under different environmental conditions (three temperatures) until their first mating. Spermatogenesis of pulmonate gastropods is sensitive to both temperature and photoperiod (Tompa, 1984). This second approach allowed us to partition the genetic variance from the total phenotypic variance and thus to estimate the broad-sense heritability $H^2$ of sperm length and shell breadth (Lynch & Walsh, 1998).

## 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 16–24 mm; Baur, 1984). Individuals become sexually mature at 2–4 years and adults live another 3–4 years (Baur & Raboud, 1988). Outcrossing is the dominant mode of reproduction in *A. arbustorum* (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 & Baur, 1993). However, the reproductive success of selfing individuals was less than 2% of that of outcrossing snails. Mating in *A. arbustorum* includes elaborate courtship behaviour, which lasts 2–18 h (Baur & Baur, 1992). Copulation is reciprocal; after intromission each snail simultaneously transfers one spermatophore, which is formed and filled with sperm during copulation (Hofmann, 1923; Baminger & Haase, 2001). Sperm are monomorphic in this species. Sperm length differed among populations (mean values of four populations: 878, 898, 913 and 939 μm), and—to a minor extent—even among individuals (Minoretti & Baur, 2006). Fertile sperm can be stored for more than 1 year (Baur, 1988a). In the field, *A. arbustorum* mates repeatedly in the course of a reproductive season. Snails deposit 1–3 egg batches, each consisting of 20–50 eggs (Baur, 1990). Multiple mating and sperm storage might promote postcopulatory processes in terms of competition among sperm from different partners, and/or selective storage and use of allosperm from the receiver (Baur, 1994; Kupfernagel, Rusterholz & Baur, 2010).

### *Sampling site and snail maintenance*

Virgin individuals (subadult snails that had not yet completed shell growth) of *A. arbustorum* were collected from an embankment along a track in the subalpine forest near Gurnigelbad in Switzerland (46°45′N, 7°28′E, elevation 1230 m a.s.l.) in spring 2001 and 2002. The snails occurred at densities of 4–8 adults per $m^2$ on the embankment (Kupfernagel *et al.*, 2010). The snails in the sampling site were connected via streams with other populations. The snails collected were kept individually in transparent beakers (6.5 cm diameter, 8 cm deep) lined with moist soil (*c.* 4 cm) at 20°C with a light:dark cycle of 18:6 h. Within 4 weeks the snails reached sexual maturity as indicated by the formation of a reflected lip at the shell aperture. The beakers were cleaned twice per week and fresh lettuce was provided *ad libitum* as food. During winter (November 2001–March 2002) the snails were allowed to hibernate in darkness at 4°C and no food was provided.

Three criteria had to be fulfilled for a snail to be considered as virgin: (1) it had to be collected before the mating season; (2) its shell growth at the time of sampling was not yet completed (indicated by absence of reflected lip); and (3) no eggs should be laid when the animal was kept isolated for 3 weeks. A previous study showed that this procedure is highly accurate for assessing virginity in snails collected from natural populations (Kupfernagel & Baur, 2011).

The snails were marked individually with letters and numbers written 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. After shell growth was completed, we measured the shell breadth of each snail to the nearest 0.1 mm using vernier callipers.

### *Repeatability of sperm length*

To assess the repeatability of sperm length in spermatophores delivered by the same individual in successive matings, we allowed snails to mate 2–4 times over two reproductive seasons. Mating trials were performed outdoors to expose snails to natural temperature and light conditions. Two randomly chosen active snails (individuals with an extended soft body and everted tentacles) were allowed to copulate in a transparent plastic container, measuring 14 × 10 × 7 cm, whose bottom had been covered with moistened paper towelling to maintain activity. Mating trials were initiated in the evening

and ran during several nights in June and July. Snails that did not mate within 8 h were tested again 3 days later with a new partner. Between trials, unmated snails were kept isolated as described above.

After copulation, one mating partner (hereafter referred to as sperm donor) was kept isolated. The other mating partner (hereafter referred to as sperm recipient) was frozen immediately after copulation in a freezer at −18°C. Sperm donors were allowed to remate with a second partner in the same reproductive season and with a third and fourth partner in the following season (all sperm recipients were virgin individuals). The recipients were dissected and their diverticulum removed to obtain the spermatophore received from the sperm donor. We measured the length ($L$) of the sperm-containing part of each spermatophore and its diameter at both ends ($D_1$ and $D_2$) to the nearest 0.1 mm using a dissecting microscope. Spermatophore volume was approximated, by the formula $V = 1/12\,\pi L(D_1^2 + D_1 D_2 + D_2^2)$, assuming a truncated-cone volume. Spermatophores were kept singly in Eppendorf tubes at −30°C until required.

The beakers of the sperm donors were checked twice per week for eggs. The eggs deposited after the first mating were collected and kept in plastic dishes (6.5 cm in diameter) lined with moist paper towelling at 19°C. The families of hatchlings were used for the breeding experiment (see below).

We assessed sperm length in all spermatophores obtained. We digitized randomly chosen sperm using a camera (SONY CCD-Iris) mounted on a 190 light microscope (Leica DMLD, magnification × 200) connected to a Macintosh computer. We measured the total length (head and tail) of 25–30 sperm per spermatophore using an image-analysis system (Minoretti & Baur, 2006), and calculated mean sperm length. Using this technique, measurements of sperm length are highly repeatable (calculated as intraclass correlation: 0.92; Minoretti & Baur, 2006).

Sperm length might be influenced by the number of sperm produced (e.g. Snook, 2005). To examine the potential trade-off between sperm length and sperm number, we counted the number of sperm in the spermatophores, following Locher & Baur (1997), in a subsample of 15 snails, which mated twice ($n = 30$ spermatophores). Briefly, the sperm suspension obtained from the mechanically disrupted spermatophore was stained with a DNA marker (gallocyanin–chromium complex). Two subsamples of known volume of the sperm suspension were transferred to a Bürker-Türk counting chamber. We counted all sperm heads in randomly chosen cells until the total number of sperm heads exceeded 400, and used the average of the two subsamples to calculate the total number of sperm transferred in a spermatophore. We adjusted our estimate of sperm number by multiplying the value with a correction factor of 1.00068, which considers the proportion of sperm removed for sperm length measurements (following Minoretti & Baur, 2006).

### *Breeding experiment to estimate heritability*

To estimate the heritability of sperm length and shell breadth in *A. arbustorum*, hatchlings of single-mated mother snails were raised at three different temperatures using a full-sibling split design, and sperm length and shell breadth were measured in individuals, which completed shell growth and attained sexual maturity. Newly hatched snails from 29 singly mated mothers ($n = 1095$) were randomly assigned to one of three temperature treatments: 11°C (mean temperature ± SD measured by data loggers: 10.7 ± 0.2°C), 15°C (14.7 ± 0.2°C) and 20°C (20.0 ± 0.2°C) in such a way that similar numbers of offspring from each family were raised at each temperature. Three climate chambers, with a light:dark cycle of 16:8 h, were used for the three temperature environments. To minimize effects of intraspecific competition, a maximum of 38 hatchlings (siblings) were kept in 750-ml plastic containers lined with moist soil. After 4 weeks, the number was reduced to a maximum of 20 juvenile snails per 750-ml container, and subsequently to 3 individuals per 225-ml container after 12 weeks. The containers were cleaned twice per week and fresh lettuce was provided *ad libitum* as food. At an age of 6 months, the snails were allowed to hibernate in darkness at 4°C for 3 months. The breeding experiment was started with 1095 hatchlings from 29 families. In spring, the 166 offspring belonging to 20 families that survived the overwintering period were kept isolated in transparent plastic beakers lined with moist soil (as described above). However, only 78 of the 166 snails reached sexual maturity in the three temperature treatments within 1 year in summer 2002 (the others attained sexual maturity later). Sixty of 78 offspring from 13 mothers (3–10 full-sibs per family) mated in the experiment. However, not all temperature treatments contained full-siblings from the same family, reducing the number of families to 10 (with a total of 48 offspring). The offspring were allowed to mate with an adult *A. arbustorum* collected in the wild in spring 2002. The spermatophores of the mating offspring were obtained by dissecting the recipients and sperm length was measured as described above. In a subsample of 19 offspring from 5 families we also assessed the volume of the spermatophore and the number of sperm delivered in each spermatophore (as described above). Adult shell breadth was measured in all offspring ($n = 48$).

### *Statistical analyses*

All statistical analyses were carried out with SPSS® version 20 (IBM® SPSS®, 2011).

Mean values ± 1 standard error are presented. Possible relationships between shell breadth, sperm length, number of sperm delivered and spermatophore volume were analysed using Pearson correlations. To examine the effects of repeated mating and individual snail on sperm length in the parent generation, we used a two-way ANOVA with the fixed factor mating and the random factor individual. Variance components for sperm length were partitioned among sources (among 27 snails, between 2–4 matings for each snail) by using a nested analysis of variance. Differences in the coefficient of variation between snails (CV, adjusted to sample size: $CV_{adj.} = (1 + \frac{1}{4}\,n)\,CV$) were examined using Levene's test.

We estimated repeatability of sperm length in successive matings following Lessells & Boag (1987). We calculated the intraclass correlation in sperm length derived from a one-way ANOVA, and adjusted it for unequal samples size ($n_o$).

To assess the influence of different environmental conditions on sperm length, offspring of 10 families were kept at three temperatures (full-sibling split design, see Methods). As a result of mortality, unbalanced sample sizes were obtained for the families and the three temperatures (11°C, $n = 13$; 15°C, $n = 16$; 20°C, $n = 19$). In the analyses we only considered families with at least one offspring at each temperature. In the case that a family had more than one offspring at a given temperature, we calculated the mean value of the offspring per family at this particular temperature. This procedure balanced the design and allowed us to use an ANOVA with temperature as a fixed factor and family of origin as a random factor. The degrees of freedom did not allow any calculation of the interaction between the factors. However, an interaction between family and temperature exists when the reaction norms, i.e. the lines connecting the sperm length of offspring of single mothers kept at different temperatures cross each other (see Fig. 1). We did not use an analysis of covariance correcting for shell breadth, because the correlations of sperm length with shell

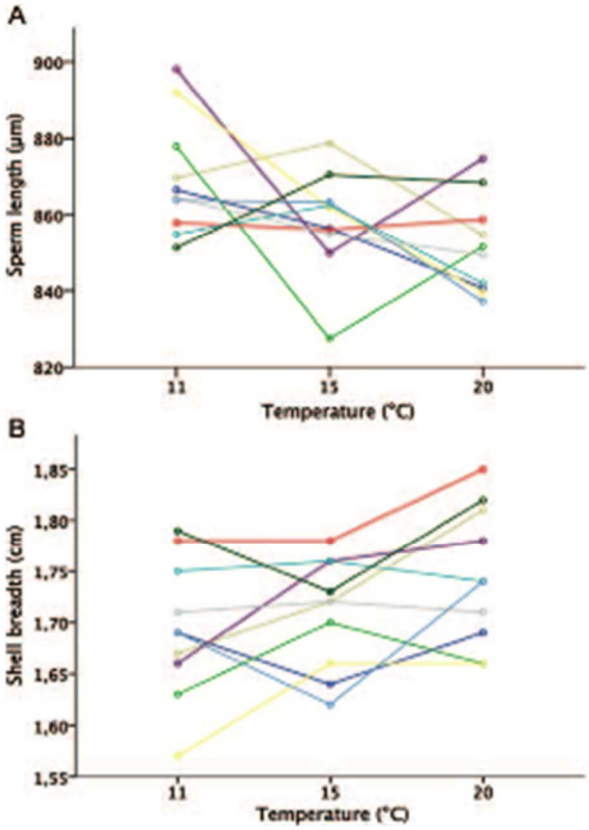


**Figure 1.** Sperm length and shell breadth of offspring of the land snail *Arianta arbustorum* raised at three temperatures. Sperm length (**A**) and shell breadth (**B**) for full-siblings of 10 families. In the case that a family had more than one offspring at a given temperature, we calculated and plotted the mean value of the offspring per family at this particular temperature.

breadth depended on the temperatures, which rendered an ANCOVA invalid (Sokal & Rohlf, 1995).

Narrow-sense heritability $h^2$ was estimated using the traditional one-parent–offspring regression, for which the level of genetic determination is given by multiplying the slope ($b$) of the regression by 2, i.e. $h^2 = 2b$, and the associated standard error equals twice the SE of the slope of the regression (Roff, 1997). This model uses information from the mother and the offspring generation, but does not consider temperature-induced variability in offspring traits. In a second approach, i.e. the full-sibling split design, we used the variance components of sperm length and shell breadth of the ANOVA to calculate $H^2$, their broad-sense heritability ($H^2$ = intraclass correlation coefficient multiplied by 2) and the associated SE following Roff (1997).

## RESULTS

### *Repeatability estimates*

Repeatability of sperm length was assessed in 27 snails that copulated 2–4 times. Mean sperm length differed among snails but not between successive matings of the same individual (inter-individual range: 844–922 µm; grand mean: 879 µm, $n = 27$; Table 1). The inter-individual variation in mean sperm length did not differ in successive matings (CV range: 1.9–2.6%; Levene's test: $df_1 = 3$, $df_2 = 63$, $P = 0.59$). Approximately half of the variation (49.1%) in sperm length can be attributed to differences among snails, 5.5% to differences between matings of the same snail and 45.4% to differences within individuals. The repeatability of mean sperm length in successive matings of a single snail was 85.9% (ANOVA: $F_{26,40} = 16.10$, $P < 0.0001$).

Mean sperm length of a snail was not correlated with its shell breadth ($r = 0.03$, $n = 27$, $P = 0.89$). The number of sperm delivered ranged from $2.0 \times 10^6$ to $3.4 \times 10^6$ (grand mean: $2.6 \times 10^6$, $n = 15$; Table 1). Mean sperm length was neither correlated with the number of sperm delivered ($r = 0.16$, $n = 15$, $P = 0.58$), nor with the spermatophore volume ($r = 0.25$, $n = 26$, $P = 0.23$).

### *Sperm length and shell breadth in offspring*

The ANOVA with a balanced design revealed that sperm length significantly decreased with increasing temperature ($F_{2,18} = 3.82$, $P = 0.042$; Fig. 1A). The variance component associated with the random factor family was not significantly different from zero. The interindividual variation measured as CV in sperm length of the offspring generation was 2.0% ($n = 48$), and did not differ from the variation found in the parent generation (1.3%, $n = 10$; Levene's test: $df_1 = 1$, $df_2 = 56$, $P = 0.13$).

Shell breadth increased significantly with temperature ($F_{2,18} = 4.61$, $P = 0.024$; Fig. 1B). The variance component of family (0.0024 or 61% of total) was significantly different from zero (likelihood ratio test: $\chi^2 = 9.6$, df = 1, $P = 0.002$) for shell breadth. The interindividual variation in shell breadth of the offspring generation was 4.3% ($n = 48$), which was similar to the value calculated for the parent generation (CV = 4.0%, $n = 10$; Levene's test: $df_1 = 1$, $df_2 = 56$, $P = 0.97$).

### *Heritability of sperm length and shell breadth*

We used two approaches to estimate the heritability of sperm length. First, the one-parent–offspring regression (following Roff, 1997) revealed an $h^2$ of 0.52 (SE = 0.55, $n = 10$; $t = 1.44$, $P = 0.16$) for sperm length. Second, we used the ratio of the genetic variance to the total phenotypic variance extracted from the ANOVA of the offspring kept at different temperatures as an estimate of $H^2$ (i.e. data from the full-sibling split design). Heritability of sperm length assessed in offspring was not significantly different from zero (in fact the estimated variance component was negative, $-0.19 \pm 0.28$). A minor part of the variance can be attributed to the family of origin (9.3%). The variance due to different temperatures was 24.4%. Unfortunately, many individuals died in the course of the experiment resulting in a low sample size. Thus, due to the low sample size, the experiment has little statistical power to test for low or moderate heritabilities.

Based on the mother–offspring regression, shell breadth showed a significant high heritability ($h^2 = 0.90$, SE = 0.33, $n = 10$; $t = 2.68$, $P = 0.012$). The father–offspring regression revealed a lower heritability than that obtained from the mother–offspring regression ($h^2 = 0.18$, SE = 0.42, $n = 10$; $t = 0.49$, $P = 0.63$). Heritability of shell breadth ($H^2$) assessed from the ANOVA of the offspring kept at different temperatures was 1.08 (SE = 0.27, $n = 3$ snails for each of the 10 families). The variance explained by the family of origin was 53.9% and that attributable to the different temperatures 21.7%.

## DISCUSSION

The present study showed that individuals of *Arianta arbustorum* delivered sperm of constant length in four successive matings. The high repeatability of sperm length suggests a genetic determination of this trait, while the results of our breeding experiment, in which full-siblings were raised at different temperatures,

**Table 1.** Summary of two-way ANOVAs examining the effects of individual snails and repeated matings on sperm traits in *Arianta arbustorum*.

| Sperm trait | Grand mean ± SE | Mean for each mating ± SE | | | | Individual snail | | | Mating | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1st | 2nd | 3rd | 4th | df | *F* | *P* | df | *F* | *P* |
| Sperm length (μm) | 879 ± 4 (27) | 878 ± 4 (27) | 881 ± 4 (27) | 865 ± 6 (7) | 859 ± 6 (6) | 26, 37 | 12.22 | <0.0001 | 3, 37 | 1.12 | 0.36 |
| Number of sperm delivered ($\times 10^6$) | 2.6 ± 0.1 (15) | 1.9 ± 0.2 (15) | 3.3 ± 0.2 (15) | – | – | 14, 14 | 0.38 | 0.96 | 1, 14 | 17.36 | 0.001 |
| Spermatophore volume ($mm^3$) | 2.7 ± 0.1 (26) | 2.9 ± 0.2 (26) | 2.5 ± 0.2 (26) | 2.5 ± 0.4 (5) | 2.6 ± 0.4 (5) | 25, 34 | 2.61 | 0.005 | 3, 34 | 1.62 | 0.20 |

Sample sizes are given in parentheses.

revealed both environmental and—to a minor extent—genetic effects on sperm length. Our results indicated that sperm length may be affected by the temperature, and this fact should be considered when studying genetic components of sperm length.

In the case of systematic maternal or environmental effects, repeatability may overestimate the corresponding narrow-sense heritability (Falconer, 1981). The high repeatability of sperm length between matings could result from the same environmental conditions to which snails were exposed in the laboratory culture. Interindividual differences in sperm length may derive from both genetic and environmental influences. We used a full-sibling split design to estimate the heritability corrected for environmental effects (Roff, 1997). A higher temperature (20°C) resulted in shorter sperm (Fig. 1A). Approximately a quarter of the variance (24.4%) in sperm length could be explained by temperature and only 9.3% by the family of origin. The sample size with the resulting degrees of freedom did not allow us to calculate the interaction between the two factors. However, an interaction between family of origin and temperature exists if the reaction norms, i.e. the lines connecting the sperm length of full-siblings raised at the three temperatures from different mothers, cross each other. Figure 1A indicates that this was the case in our breeding experiment. In pulmonate gastropods, spermatogenesis is sensitive to both temperature and photoperiod (Tompa, 1984). For example, the rate of spermatogenesis in *Helix aspersa* decreased at temperatures below 15°C and stopped at 5°C. At temperatures of 20–25°C, the multiplication of sperm cells and differentiation of spermatozoa proceeded within 3–4 weeks (Gomot de Vaufleury, 2001). Similar information is not available for *A. arbustorum*.

We used two different approaches to determine the level of genetic determination of sperm length. The one-parent–offspring regression revealed a relatively high estimate of heritability ($h^2 = 0.52$) but this value was not significantly different from zero. The parent–offspring regression reflects the influence of the genes transmitted from parents to their offspring combined with environmental effects (Roff, 1997). In the second approach, we used the ratio of the genetic variance to the total phenotypic variance extracted from the ANOVA of the offspring kept at different temperatures as an estimate of $H^2$, the broad-sense heritability. Based on families of full-siblings, this approach revealed a heritability estimate, which was not significantly different from zero. Thus, the data suggest relatively little genetic variation in sperm length in the studied population, because both approaches indicate almost no statistically significant resemblance between related individuals. However, due to the small sample size, the absence of a family effect or of a significant slope from the parent–offspring regression is only suggestive of an absence of a very strong genetic effect.

There are at least three explanations for the different heritability values of sperm length obtained by the two approaches. First, the sample size of offspring was relatively small. We started the breeding experiment with 29 families and 1095 hatchlings. However, only 10 families with 48 offspring could be considered in the analyses (see Methods). In our experiment, juvenile survival (15.2% after first hibernation) was approximately twice as high as recorded in natural populations of *A. arbustorum* (7.6%; Andreassen, 1981; Akçakaya & Baur, 1996). This demonstrates that huge breeding stocks are required to receive adequate sample sizes for experimental heritability estimates in traits with low genetic determination. The negative heritability indicates that offspring deviated consistently and in the opposite direction from the population mean of their parents (Palmer, 2000). As sample size decreases, the likelihood of obtaining a negative heritability (<0) or an extremely positive (>1) heritability increases substantially (Palmer, 2000).

Second, the heritability estimate obtained in the full-siblings split design is based on the ratio of the genetic variance to the total phenotypic variance. The total phenotypic variance includes the variance among individuals resulting from environmental differences. Therefore, an increase in the environmental variation (temperature) decreases heritability (Hartl, 2000).

Finally, we did not consider offspring that reached sexual maturity after one year. Thus, our heritability estimate is based on a subsample of fast growing snails. Individual growth rate is known to influence adult size in *Cepaea nemoralis* (Oosterhoff, 1977). However, it is not known whether growth rate affects sperm length in terrestrial gastropods.

The adaptive significance of sperm length variation is still unknown in *A. arbustorum*. Post-copulatory mechanisms of sexual selection could be a selective force for sperm length evolution (Pitnick *et al.*, 2009a). In the wild, sperm length differs among populations of *A. arbustorum* and even among individuals (Minoretti & Baur, 2006). In snails from two populations, no correlations between sperm length, velocity, percentage motility and longevity of sperm were found (Minoretti & Baur, 2006). Spermatozoa received from the mating partner are stored in the blind-ending tubules of the spermatheca, attached by the heads to the spermathecal epithelium (Bojat *et al.*, 2001). Rogers & Chase (2002) suggested that the unified beating of the flagella of sperm from the first mating provide resistance to incoming sperm from subsequent matings entering the tubules and in this way function as a paternity assurance. It is possible that longer sperm provide an increased resistance to incoming sperm, which would increase their chances for fertilization success. This hypothesis needs to be tested.

Shell breadth of offspring was significantly affected by both the family of origin and the temperature treatment. A higher temperature resulted in larger adult shells (Fig. 1B). The parallel-running reaction norms in Figure 1B suggest that no genotype–environment interaction occurs. Both methods revealed high heritabilities for shell breadth in *A. arbustorum* (one-parent–offspring regression: $h^2 = 0.90$; offspring estimate: $H^2 = 1.08$). The latter, as discussed above, may be a result of

the relatively small sample size, which can lead to an extreme positive (>1) heritability. Thus, offspring exhibit consistently more extreme phenotypes than their parents (Palmer, 2000). On the other site, heritability estimates derived from full-sibling split design that are larger than 1 more likely indicate that some of the genetic variance is due to dominance, epistasis or common environment in early life (Simmons & Moore, 2009). However, the effect of the family of origin was so strong that it was significant even with a small sample size. Our results confirmed the relatively high heritability of shell breadth in pulmonate land snails (*A. arbustorum*: 0.70 (Cook, 1965) and 0.54 (Baur, 1984); *Partula taeniata*: 0.40 and *P. suturalis*: 0.53 (Murray & Clarke, 1968)).

We also assessed the heritability of shell breadth using separate one-parent–offspring regressions for both mother and father snails. Interestingly, $h^2$ of shell breadth estimated with the father-offspring regression was 0.18, i.e. 5 times smaller than that of the mother–offspring regression ($h^2 = 0.90$), suggesting a maternal effect on shell size. This result is of importance because female reproductive traits including egg size and clutch size are positively correlated with shell size in *A. arbustorum* (Baur 1988b, 1990; Baur & Raboud, 1988; Baur *et al.*, 1998) as well as in other helicid snails (Dupont-Nivet *et al.*, 2000).

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# General discussion

The aim of this thesis was to investigate life-history traits, sex allocation strategies and sexual selection mechanisms in the simultaneous hermaphrodite land snail *Arianta arbustorum*. Sexual selection can be considered to consist of a number of components that affect total fitness, with two major routes: mating success and fecundity per mate (Møller, 1998). In hermaphrodites, sex allocation simply represents a decision about how resources are allocated to different organs and behaviours within an individual. Therefore, and in contrast to gonochorists, sex allocation will influence the immediate reproductive success of the individual rather than that of its offspring (Borgia & Blick, 1981; Michiels, 1998; Michiels *et al.,* 1999).

Sex allocation theory aims to predict the optimal sex allocation that an organism should exhibit under different environmental and social conditions, which makes it a central topic in life-history theory (Charnov, 1982; Stearns, 1992; De Jong & Klinkhamer, 2005). Thus, how are resources allocated to different organs and behaviours within an individual given certain environmental conditions? Effects of soil type on reproductive traits have so far received little attention in terrestrial gastropods. In **Chapter I,** we showed that soil type could affect mating propensity, female but not male reproductive traits in *A. arbustorum*. Unexpectedly, the total number of eggs produced was larger in snails kept on Ca-poor soil than in individuals maintained on Ca-rich soil. The resulting Ca-uptake per week that a snail might have invested in egg production was probably obtained by the lettuce consumed and/or by compensatory feeding. Thus, the snails kept in the Ca-poor soil received enough Ca to allow egg production. On the other hand, there may also be a trade-off between reproductive output and survival. Snails living in environmentally stressful conditions may allocate more resources into reproduction in the first reproductive season but may die earlier than those living in more favourable conditions. This hypothesis could be tested by maintaining snails over two or more years under the experimental conditions of the present study. However, snails kept in the Ca-rich soil had a reduced mating propensity and reproductive output. Apart from Ca availability, most probably other soils parameters may influence the reproductive output of *A. arbustorum*. This study examined – to our knowledge for the first times – soil-related effects also on male reproductive output. However, neither the number of sperm delivered nor spermatophore size differed between the two snail groups kept in different soils.

How do behavioural and genetic traits influence mate choice, and female and male reproductive success? In the study presented in **Chapter II**, we used a combination of behavioural and genetic data collected in groups of snails kept in a semi-natural environment over one reproductive season. We found 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. 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 a higher winter mortality than parasite-free snails (Schüpbach & Baur, 2010). Consequently, a high activity and healthiness result in a large number of mate encounters, and thus influence reproduction in this simultaneous hermaphrodite. The extended courtship in pulmonate land snails should provide ample opportunities for partner assessment and/or mate choice (Baur, 1998). In our study, 2.2% of the long contacts (interindividual range 0–13.7%) led to courtship, and 60.3% of the courtships (interindividual range 0–100%) resulted in copulation, suggesting a multilevel assessment of potential partners in *A. arbustorum*, although the relevant cues are not known. 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, 2000a). In our study, the degree of heterozygosity explained variation in mating success and in female and male reproductive success.

We also 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 semi-natural 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, so far there is little empirical evidence for this trade-off in animals (Schärer, 2009). Locher & Baur (2000b) 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 (2000b) 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 snail.

Sexual selection theory attempts to explain the evolution of anatomical, physiological, and behavioural adaptations associated with reproduction. In postcopulatory processes of sexual selection, male success may be skewed at the time of fertilisation if females favour the sperm of certain males over others, and if the sperm of a male are competitively superior, or if reproduction depends on the phenotype of the mate (Møller, 1994). Do snails differ in sperm quality characteristics that in turn may play a role

in sperm competition? In gastropods, the interspecific variation in sperm morphology has been studied, and is used as a taxonomical character (e.g. Healy, 1996), while the intraspecific variation in sperm traits has not yet been analysed quantitatively. Spermatozoa of terrestrial gastropods are among the longest within molluscs (e.g. 850 µm in *Helix pomatia*, and 1140–1400 µm in *Hedleyella falconeri*; Thompson, 1973). **Chapter III** focuses on intraspecific variation in sperm characteristics in the simultaneously hermaphroditic land snail *Arianta arbustorum*. In this species, sperm are monomorphic and ca. 800 µm long (Bojat *et al.*, 2001). We found significant differences in sperm length, both among- and within-populations of *A. arbustorum*. Sperm length was not affected by snail size and the weight of the albumen gland (a measure for female allocation). This suggests that sperm length in *A. arbustorum* is not dependent on allometry or on body conditions, confirming similar studies on gonochoristic animals (e.g. Hellriegel & Blanckenhorn, 2002; Schulte-Hostedde & Millar, 2004).

Sperm competition models predict that sperm size can confer a fertilization advantage, but only under the assumption of a functional relationship between sperm quality traits (e.g. sperm velocity, motility and longevity). Theory for internally fertilizing species predicts that enhanced sperm competition risk would favour increased sperm length when larger sperm enjoy higher survival, and could be stored until fertilization (Parker, 1998). In *A. arbustorum*, the longer sperm of snails from one population survived longer than the shorter sperm of snails from the other population considered in our study. Within populations, however, our study did not find any relationship between sperm length and longevity. In contrast to theoretical models (Katz & Drobnis, 1990), sperm length and sperm velocity were not associated. Moreover, sperm velocity was not correlated with sperm longevity. The effect of sperm motility on fertilisation success has not yet been examined. Roger & Chase (2002) suggested that in the snail *Helix aspersa* the beating of longer sperm should generate resistance to incoming sperm of rivals in the sperm storage organ.

Sexual selection on sperm length requires a significant additive genetic variance; i.e. the trait must be heritable. In *A. arbustorum*, is there the potential for evolution to select individuals according to the length of their sperm? Sperm size usually exhibits little variation across ejaculates of single males (Morrow & Gage, 2001a; Birkhead *et al.*, 2005; Immler *et al.*, 2008) indicating a strong genetic determination (Morrow & Gage, 2001b; Simmons & Kotiaho, 2002). However, there is some evidence that environmental factors also may influence sperm length (e.g. temperature, larval density, nutrition; Blanckenhorn & Hellriegel, 2002; Morrow *et al.*, 2008; Amitin & Pitnick, 2007). Furthermore, sperm length may partly be determined by maternal effects (Dowling *et al.*, 2007; Gay *et al.*, 2009) and by males'age (Green, 2003). In **Chapter IV**, we present the first estimates of heritability of sperm length in a Stylommatophoran gastropods. The study showed that individuals of *A. arbustorum* delivered sperm of constant length in four successive matings. The high repeatability of sperm length suggests a genetic

determination of this trait. However, the results of our breeding experiment, in which full-siblings were raised at different temperatures, revealed both environmental and – to a minor extent – genetic effects on sperm length. Our results indicated that sperm length may be affected by the temperature, and this fact should be considered when studying genetic components of sperm length.

In **Chapter IV**, we also assessed the heritability of shell breadth using separate parent-offspring regressions for both mother and father snails. The effect of the family of origin was so strong that it was significant even with a small sample size. Interestingly, $h^2$ of shell breadth estimated with the father-offspring regression was 0.18, i.e. 5 times smaller than that of the mother-offspring regression ($h^2$ = 0.90), suggesting a maternal effect on shell size. This result is of importance because female reproductive traits including egg size and clutch size are positively correlated with shell size in *A. arbustorum* (Baur 1988b, 1990; Baur & Raboud, 1988; Baur *et al.*, 1998) as well as in other helicid snails (Dupont-Nivet *et al.*, 2000).

## OUTLOOK

In this thesis, we studied sex allocation in a simultaneous hermaphrodite, in which sex allocation decisions that affect reproductive success take place within an individual. Sex allocation models predict a fixed sex allocation for all individuals within a population (Schärer, 2009). However, we found that individuals could make short-term adjustments in sex allocation in response to current conditions (i.e. to Ca/soil type in our study). Thus, phenotypic plasticity should be considered in the models. However, we showed that female but not male allocation changed. While we confirmed some of the assumptions (e.g. the non-linearity of the male fitness gain curve) and some of the predictions (e.g. sex allocation is in general female-biased, or sex allocation varies with body size) of sex allocation theory, other patterns did not match and we even found differences between some of the central assumptions of theoretical models (e.g. the absence of the sex allocation trade-off). We found a positive correlation between female and male reproductive success during a reproductive season, but it is an open question how resource allocation translates into fitness. Moreover, future 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.

Snails often mate multiply, most frequently with different partners. *A. arbustorum* individuals may obtain direct benefits from mating with multiple partners. It would be interesting to study mate choice decision based on indirect (i.e. genetic) benefits resulting in higher quality offspring and, in particular, to test if female mate preferences favour genetically dissimilar mates. Our study design did 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. In relation to the mechanism underlying mate choice

processes in *A. arbustorum*, it would be interesting to look for the empirical evidence of (chemical) cues for partner assessment.

In this work, we consider sperm quality traits, which are important factors in postcopulatory sexual selection for other species. Our data did not support any trade-off between sperm size (total length) and sperm function (velocity, motility and longevity). Thus, these findings did not bear out the assumptions of sperm competition models on the evolution of sperm size for internally fertilizing species like *A. arbustorum* (Parker, 1998). Future research should examine genetic correlations between sperm traits to elucidate the constraints on the evolution of sperm morphology and function.

Interindividual differences in sperm quality traits found in *A. arbustorum* could be a response to sperm competition risk in interaction with cryptic female choice. The adaptive significance of sperm length variation in *A. arbustorum* should be investigated in laboratory experiments. It remains to be determined whether snails with long sperm actually enjoy an increased fertilization success in multiple mated snails. It could also be interesting to investigate the mechanisms that make the sperm of an individual more successful in fertilisation than the sperm of rival males. Further work is needed to assess the energy supply of *A. arbustorum* sperm (ATP and/or glycogen content). For example, in domestic fowl, longer sperm have increased motility because of their higher ATP/mitochondria. On the other hand, sperm with a higher energy supply may survive longer before fertilisation and thus enhance individual fertilisation success.

In general, this work underlines that reproductive traits (and also body size) in *A. arbustorum* are likely to be shaped by a complex set of genetic and environmental factors that affect populations to different degrees and that have probably different magnitudes over time. An intriguing aspect of our results is that environmental conditions influenced snail activities to a different extent, and in turn different environments might result in different levels of multiple paternity in *A. arbustorum*. Some reproductive traits were also influenced by the population of origin of the snails and by shell size emphasizing the importance of proper design and replication of life-history studies in gastropods.

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