The influence of sperm competition and parasites on reproductive strategies in simultaneously hermaphroditic land snails

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

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aus

Tübingen, Deutschland

Bremen, 2016

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag von

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Basel, den 24.05.2016

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(Dekan)

Acknowledgements

First, I would like to express my deep gratitude to Prof. Bruno Baur for the opportunity to realize my PhD thesis in this fascinating research topic in his research group at the University of Basel, Switzerland. I am also very thankful for his field assistance in his free time and his continuous support, efforts and expert knowledge during data analyses and publication.
Furthermore, I am deeply thankful to Denes Schmera for all his help and his statistic genius.
I am very grateful to Anette Baur for her field assistance during weekends and for the proofreading of all manuscripts.
I wish to thank Sylvain Ursenbacher for his efforts and support in the laboratory.
My special thanks go to Julia Pizá, who joined and supported me since 2010. It was always a pleasure to work and spend time with her. Her diligence and her love for gastropods and biology itself inspired me. Thank you my friend!
I want to thank Eliane Riedener, Sarah Reiling and Sebastiaan Wiering for their valuable friendship (!) and their helpful comments on my final script.
My special thanks are extended to Prof. Andreas Erhardt and Prof. Thomas Boller, who kindly accepted to be member of my thesis committee.
I would like to thank all people from the NLU – current and former – for company and their help in every occasion. Here I would like to mention Regina Ruckli, Samuel Zschokke, Silvia Geser, Matthieu Raemy and Ruben Janssen. Thank you for the good times!
I wish to thank all people outside the NLU, who offered their field assistance and Georg FJ Armbruster for his help to identify Clausilidae and other tricky snail species.

Financial support was received from the Swiss National Foundation.

Finally, I would like to express my very great appreciation to my family and close friends. My deep gratitude goes to my parents, Mercedes and Peter Häußler, my sister Hanna Häußler and her beloved Sven, my grandmothers Renate and Ruth, Wolfgang, Suse and Bonkel Jü for their love, patience, never ending support and encouragement throughout my whole life. I love you too!
I want to thank my dear friends Julia Boschmann, Tobias Simon, Hanna Mittag, Julia Binder, Wolfgang Meierhoff, Petra Bareiss, Evelyn Krisch and Betty Kolodzy for their support, encouragement and open ears. I cannot express how much I value your friendship!
Last but not least, I am much obliged to my husband Florian Reinartz for his love, support, encouragement, endless patience and all the good times we had.

Contents

Summary

Sexual selection is a concept of selection and subject of numerous studies on reproduction and sexual dimorphism in secondary sexual characteristics. In simultaneous hermaphrodites both the female and male function within the same individual compete for resources. Complex behaviours and mechanisms optimize the reproductive investment in these species. These behaviours and mechanisms might also be affected by internal factors and external conditions. In former studies researchers started to disentangle different factors and conditions in order to understand the major drivers of reproduction success. In my thesis I focused on reproductive strategies of the model organism *Arianta arbustorum*, a hermaphroditic land snail, and the influence on reproduction and winter survival of its parasite *Riccardoella limacum*, a blood-sucking mite that lives in the mantle cavity of the snail.

For a better understanding of the fragile host-parasite interaction, further details about the life-history of *R. limacum* were explored. Hibernation is a crucial period in the annual cycle of a parasite and its host. Hibernation has a strong impact on the fitness of both species and consequently affects their life-history and reproduction. Temperature, for example, can be a limiting factor for parasite development. Low temperatures over a long period could influence the life cycle of *R. limacum*. Three experiments were performed to examine the influence of hibernation duration on the survival of the parasitic mite *R. limacum* and its eggs. The intensity of mite infection of hosts decreased with increasing hibernation duration. *R. limacum* survived the winter in the egg stage in the host's lung. Winter survival of the host itself was negatively affected. It could be hypothesised that low temperature and a longer winter period at high elevation may limit the occurrence of *R. limacum*.

The variation in sperm morphology of the land snail *Arianta arbustorum* in relation to parasitic mite infection was investigated. Variation in total sperm length and sperm head length was assessed in 23 populations sampled across the distributional range of the species in Central and Northern Europe. Results showed a variation in total sperm length among populations, which increased significantly with the geographic distance between the populations. Additionally, a minimal adequate model revealed that mite-infected individuals of *A. arbustorum* produced longer sperm than uninfected snails and total sperm length decreased from west to east across Europe. Moreover, total sperm length in a subsample of 12 alpine populations decreased with increasing elevation. Differences among populations explained 62.9% of the variance in total sperm length, differences among individual snails

within population 23.4% and differences within individual snails 13.7%. These results suggest that selection pressures acting among populations may differ from those acting within.

To examine the principles of selection pressures on sperm traits on a larger scale, a comparative approach was used considering 57 stylommatophoran snail species of Europe and South America. The hypothesis that cross-fertilizing species have longer sperm than self-fertilizing species as a consequence of sperm competition risk was tested. Sperm length, sperm head length and shell traits of these species were measured and analysed. Information on the breeding system and life-history characters including habitat preferences was gathered. Results showed that both total sperm length and sperm head length varied significantly between species and indicated that risk of sperm competition as well as shell size account for the variation across species.

Sperm competition is an important part of sexual selection and considered as a driving force in the evolution of sperm traits. Furthermore, sperm competition is regarded as principal determinant of male fitness in promiscuous species. The last project of this thesis deals with the question, whether the risk of sperm competition leads to a preference for mating with virgin individuals. In promiscuous species with sperm storage and multiple paternity, males are expected to prefer mating with a virgin partner to assure an exclusive paternity. In a series of mate-choice tests I examined whether virgin and nonvirgin individuals of *A. arbustorum* discriminate between virgin and nonvirgin mates. I also measured the sperm number of the ejaculates delivered to virgin or nonvirgin partners to see whether the snails adjust sperm delivery. In all experiments mate-choice was random and the number of sperm delivered was not adjusted to the mating status of the partner. The mating success seemed to be determined by the activity of the individual snail and its partner. Random mating does not imply a random fertilization of eggs. The presence of a sperm-digesting organ and the morphology of the sperm storage organ allow a selective storage and use of sperm in *A. arbustorum*.

This thesis showed that reproductive strategies and sperm traits in simultaneously hermaphrodites are influenced by species' morphology, environmental conditions and parasitic infection.

General introduction

When Antonie van Leeuwenhoek (1632–1723) published his first description of sperm (Leeuwenhoek 1678), the foundation was laid for centuries of research on sperm characteristics and their role in sexual selection. Van Leeuwenhoek was the first researcher to measure sperm motility and longevity, and he described motion of the flagellum and commented on differences in sperm appearance. Two hundred years later, Darwin‘s ideas in his books “The origin of species” (1859) and “The decent of man and selection in relation to sex” (1874) caused large-scale changes in the way of thinking. Previously, reproduction was seen as a beneficial arrangement between males and females. Through Darwin‘s thoughts scientists started to recognize that selection could affect females and males separately and differently (Birkhead, Hosken & Pitnick 2009). Until today, scientists such as Geoff Parker (*1944) examine sperm competition and its evolutionary consequences. Parker detected the phenomenon of “male precedence” in dung flies and described that males compete for fertilizations and not *per se* for females. He proved the existence of sperm competition and showed that sexual selection does not stop at mating but it continues to fertilization (Parker 1970, Parker, Baker & Smith 1972).

Mechanisms of mate choice are of particular interest in simultaneously hermaphroditic invertebrates, in which selection for female reproductive behaviour is not independent from selection for male reproductive behaviour. Simultaneous hermaphrodites have to balance male and female investment and, therefore, have an exceptional position within sexual selection framework. On the basis of complex behavioural, physiological and biochemical mechanisms they are able to select before (mate choice) and after mating. Post-copulatory selection is the sexual selection that takes place after mate choice and copulation in the form of sperm competition and cryptic female choice (Charnov 1996, Michiels 1998, Leonard 2006). Sperm competition occurs when sperm from different males compete to fertilize the same set of ova (Parker 1970, 1998, Simmons 2001) and cryptic female choice is defined as non-random paternity biases resulting from female morphology, physiology or behaviour that arises after copulation (Pitnick & Brown 2000). Both sperm competition and cryptic female choice profoundly affect sperm morphology and advance sperm diversity within species and individuals (Holman & Snook 2006). A variety of other factors can influence reproduction and sperm morphology. There is some experimental evidence that temperature can influence sperm length in invertebrates (e.g. in yellow dung flies, Hellriegel & Blanckenhorn 2002).

Furthermore, reproduction depends on the condition of the individual. Parasites have a detrimental influence on the condition and reproductive success of their host (Agrawal 2006, Schüpbach & Baur 2008). To date, the influence of parasites on ejaculate qualities and sperm length in invertebrates is not fully understood. Therefore, the work presented here examines these mechanisms in more detail. To investigate the influence of sperm competition and parasites on a stylommatophoran species, *Arianta arbustorum* (LINNAEUS 1758) is a valuable model organism. This snail species is widely spread in different climate zones across Northwestern and Central Europe. *A. arbustorum* is commonly found in moist habitats reaching elevations up to 2700 m (Kerney & Cameron, 1979). Research on this snail was carried out for decades, and numerous studies on life history and reproduction were published (https://conservation.unibas.ch/team/baur/publications.php?lang=de).

This snail has determinate growth (shell breadth of adults 16–22 mm). Individuals become sexually mature at 2–4 years, and adults usually live another 3–4 years (maximum 14 years; Baur & Raboud 1988). Individuals mate repeatedly with different partners in the course of a reproductive season and are able to store fertile sperm for more than 1 year (Baur 1988, 2007). In the field, snails deposit one to three egg batches consisting of 20–50 eggs per reproductive season (Baur 1990). 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 transfers simultaneously one spermatophore (Haase & Baur 1995). The spermatophore is formed and filled with sperm during copulation. It has a distinctive form consisting of a head, a body (sperm container with 800 000–4 000 000 spermatozoa) and a tail that is 2–3 cm long (Baur, Locher & Baur 1998). Mating is random with respect to shell size and different degrees of relatedness (Baur 1992, Baur & Baur 1997). Outcrossing is the dominant mode of reproduction, but self-fertilization may occur in low frequencies, particularly after long periods of isolation (Chen 1994). *Arianta arbustorum* is a common host of the hematophageous mite *Riccardoella limacum* (SCHRANK 1776) (Baur & Baur 2005).

FOCUS OF THE THESIS

In **Chapter I,** the survival of the blood-sucking mite *Riccardoella limacum* is analysed during hibernation of its host *Arianta arbustorum*. In land snails, hibernation is an overwintering strategy characterized by a reduction of feeding, locomotion and reproduction (Ansart et al.

2001). Parasite-host dynamics may be affected by various factors such as environmental stress and health of the host. For example, a poor health of the host *Lymnaea stagnalis*, a fresh water snail, has a negative effect on parasite reproduction and survival of the parasitic trematode *Diplostomum spathaceum* (Seppälä et al. 2008). The conditions for parasites may also be altered during the hibernation of their host. This study focused on the influence of hibernation duration in experimentally infested host snails on the intensity of infection and host survival. For this purpose, three experiments were conducted.

Exp.1: Uninfected snails of one population were infested with mites of a naturally infected snail population. Infected snails and those of the control group were randomly assigned to four groups of about 30 individuals that were allowed to hibernate 4, 5, 6, and 7 months in a climate chamber.

Exp.2: 45 individuals of a naturally infected population were randomly assigned to seven groups and allowed to hibernate for 0 (control), 4, 8, 12, 24, 48, and 72 days. After hibernation, snails were immediately frozen, dissected and the number of mites and eggs in the lung tissue was determined to investigate the parasites' short-time response.

Exp.3: Beakers with soil of a) experimentally infected snails, b) naturally infected snails and c) control were transferred to hibernation conditions for 7 months. After that period, uninfected snails were placed singly on these three soil types and kept for three months. After three months, snails were frozen and a screening of mites and eggs in their lung tissue was performed.

In **Chapter II,** I examined the geographical variation of sperm traits and the influence of *Riccardoella limacum* on sperm length in the hermaphroditic land snail *Arianta arbustorum*. Therefore, I measured and analysed total sperm length and sperm head length of 23 populations across the distribution range of *A. arbustorum* in Central and Northern Europe.

Sperm size and shape are highly variable both within and among species (Snook 2005, Manier & Palumbi 2008, Pitnick, Hosken & Birkhead 2009). This variation may reflect population- and species-specific differences in fertilization mode, allometry and strength of post-copulatory sexual selection. Within species, stabilizing selection may retain sperm morphology (Parker 1993, Calhim, Immler & Birkhead 2007) but it can respond to directional selection, including selection pressure from the female reproductive tract (Morrow & Gage 2001, Miller & Pitnick 2002). Hence, if spatially segregated populations vary in strength or type of selection, sperm may be selected towards different optima across populations. However, information on geographical variation in sperm length is very limited.

R. limacum is a blood-sucking (Baker 1970b) ectoparasite mite that lives in the mantel cavity of its host (Baur & Baur 2005, Fain & van Goethem 1986, Turk & Phillips 1946). Graham (1994) and Graham et al. (1996) investigated the life cycle and influence of *Riccardoella limacum* on its host under laboratory conditions in its natural host *Cornu aspersum* (MÜLLER 1774). At a temperature of 20°–25°C the complete life cycle of *R. limacum* is 19–23 days. Female mites lay their eggs in the lung tissue of the host. After hatching, mites develop with three nymphal stages to sexually mature females and males of 0.4 mm body length (Baker, 1970a). *R. limacum* shows different prevalence and intensity of infection in natural populations of *Arianta arbustorum* in Switzerland (Baur & Baur, 2005). A parasite infection reduces fecundity (Jaenike et al. 1995), egg production and an increased mortality in snails (Schüpbach & Baur 2008). Classical sex allocation theory predicts an increased male allocation by producing more or larger sperm when there is a trade-off between male and female functions and a fixed amount of resources (Charnov 1982, 1996). Parasite coevolution and its consequences for sexual selection have been intensively investigated (reviewed by Agrawal 2006). Parasites may alter both the mating behaviour and mating frequency of male hosts (Moore 2002). Few studies, however, have attempted to examine parasite influences on ejaculate qualities and sperm length (Karr, Swanson & Snook 2009, Yan & Stevens 1995). To my knowledge, the influence of *Riccardoella limacum* on sperm length has not been investigated to date.

In **Chapter III,** the hypothesis that the breeding system and shell characteristics affect sperm length in stylommatophoran gastropods was tested. Aim of this study was to examine whether hermaphroditic, self-fertilizing species produce shorter sperm than cross-fertilizing species, which use sperm stored from several mating partners for the fertilization of their eggs and therefore are exposed to intense sperm competition. Additionally, the potential impact of the shape of shells (oblong, globose or depressed) was examined. Phylogenetically generalized linear models were used to explore how shell shape, age at sexual maturity, longevity, mode of reproduction (oviparous or ovoviviparous) and habitat preferences may explain sperm characteristics. For this, 57 terrestrial gastropod species (50 land snail and 7 slug species), representing 23 families of the subclass Stylommatophora were collected at various locations in Europe and South America. Total sperm lengths were measured and analysed. A phylogenetic analysis was made to determine phylogenetic relationships between the gastropod species examined.

In **Chaper IV,** the mating behaviour of the hermaphroditic land snail *Arianta arbustorum* is analysed. Promiscuity and multiple paternity are well documented in simultaneously hermaphroditic land snails (Baur 1994, Rogers & Chase 2002, Evanno et al. 2005, Garefalaki et al. 2010). Therefore, males are expected to show a preference for mating with virgin and young females to reduce the risk of sperm competition, because virgins do not yet store sperm from other males (Parker 1998). In various insect species, males prefer to mate with virgin or young females to reduce sperm competition risk for their own sperm and, therefore, increase siring success (Simmons et al. 1994, Bateman & Ferguson 2004). There is further evidence that males may provide smaller ejaculates or terminate copulation earlier when mating with females with a higher risk of sperm competition (Bukowski & Christenson 1997). Additionally, virgin females are expected to be less choosy than females who already mated (Halliday 1983).

Mate-choice-tests were performed in different combinations of virgin (V) and non-virgin (N) focus snails and partners (four test groups: VVV, NNN, VVN, NVN) equally distributed over 10 nights in altogether 196 trials.

An overview of the results is given in the "**General Discussion**". Furthermore, this section includes a discussion of findings of all four chapters to point out which factors can exert influence on reproduction, especially on sperm traits.

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Chapter I

Intensity of parasitic mite infection decreases with hibernation duration of the host snail

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Parasitology, 2012, **139**: 1038–1044

Intensity of parasitic mite infection decreases with hibernation duration of the host snail

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(*Received 24 November 2011; revised 2 February 2012; accepted 5 February 2012; first published online 23 March 2012*)

SUMMARY

Temperature can be a limiting factor on parasite development. *Riccardoella limacum*, a haematophagous mite, lives in the mantle cavity of helicid land snails. The prevalence of infection by *R. limacum* in populations of the land snail *Arianta arbustorum* is highly variable (0–78%) in Switzerland. However, parasitic mites do not occur in host populations at altitudes of 1290 m or higher. It has been hypothesized that the host's hibernation period might be too long at high elevations for mites and their eggs to survive. To test this hypothesis, we experimentally infected snails and allowed them to hibernate at 4 °C for periods of 4–7 months. Winter survival of host snails was negatively affected by *R. limacum*. The intensity of mite infection decreased with increasing hibernation duration. Another experiment with shorter recording intervals revealed that mites do not leave the host when it buries in the soil at the beginning of hibernation. The number of mites decreased after 24 days of hibernation, whereas the number of eggs attached to the lung tissue remained constant throughout hibernation. Thus, *R. limacum* survives the winter in the egg stage in the host. Low temperature at high altitudes may limit the occurrence of *R. limacum*.

Key words: *Arianta arbustorum*, *Riccardoella limacum*, winter survival, population growth, climate warming.

INTRODUCTION

Parasites are known to influence the life history, behaviour and demography of host populations (Agnew *et al*. 2000; Moore, 2002; Poulin, 2007). Hosts in turn have evolved a wide range of adaptations to prevent initial parasite infection and subsequent proliferation. These adaptations range from the extraordinary complexity of antigen-specific cell-mediated responses found in vertebrates to the simpler humoral and cell-mediated responses of invertebrates, through barrier mechanisms, to behaviour that may reduce the likelihood of an individual becoming exposed to a parasite or that may act to control infections once they have been developed (Sheldon and Verhulst, 1996; Schmid-Hempel, 1998; Moore, 2002). In parasite-host systems with invertebrate hosts and relatively large parasites, the reproduction of parasites highly depends on the condition of the host because parasites consume a high proportion of the host resources (Seppälä *et al*. 2008). For example, starvation in *Daphnia magna* (Straus 1820) reduces the spore load and prevalence of the microsporidian gut parasite *Glugoides intestinalis* (Chatton 1907), but causes also an increased host mortality (Pulkkinen and Ebert, 2004).

The conditions for parasites may also be altered during the hibernation of ectothermic hosts. This is of particular interest when the duration of the hibernation period varies among host populations. If the hibernation of the host is extremely long, then the host-parasite dynamics might be disturbed. So far, the influence of hibernation on the host-parasite dynamic has been poorly studied in invertebrates (Tyler and Jones, 1974; Callait and Gauthier, 2000; Moore, 2002; Schüpbach and Baur, 2010*a*). In land snails, hibernation is an overwintering strategy characterized by a reduction of feeding, locomotion and reproduction (Ansart *et al*. 2001). Many species bury in the soil or remain in protected places and close the shell aperture with the epiphragm, a mucoproteinacaeous membrane. The metabolism slows down, and a reduction of oxygen consumption and rate of water loss occurs (Banhart and McMahon, 1988; Ansart *et al*. 2001, 2002).

Riccardoella limacum (Schrank 1776) is an ectoparasite mite of terrestrial gastropods (Fain and Van Goethem, 1986). The mite feeds on blood in the mantle cavity of its host (Baker, 1970*a*). It reduces the activity and growth rate of the gastropod *Cornu aspersum* (Müller 1774) delaying reproductive development (Graham *et al*. 1996). In the land snail *Arianta arbustorum* (Linnaeus 1758), parasite infection reduced the activity of individuals and their

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doi:10.1017/S0031182012000327

reproductive output, and decreased winter survival (Schüpbach and Baur, 2008*a*). Host survival during hibernation decreased with increasing parasite load and differed among families, but was not affected by snail size (Schüpbach and Baur, 2010*b*). The mites are transmitted through physical contact among the hosts (Baur and Baur, 2005), via mucus trails of the host snail or indirectly via soil (Schüpbach and Baur, 2008*b*). It is unknown, however, how long the parasites can survive in the soil. Natural snail populations vary widely in prevalence of mite infection (0–78% in 11 populations; Baur and Baur, 2005). However, parasitic mites have not been found in snail populations situated at elevations of 1290 m or higher. It has been hypothesized that the host's hibernation period might be too long at high elevations for mites and their eggs to survive (Baur and Baur, 2005).

The aim of our study was to investigate the influence of hibernation duration in experimentally infected host snails on the intensity of infection and host survival. In particular, we addressed the following questions. (1) Is host survival affected by the intensity of infection and hibernation duration? (2) Does the intensity of parasite infection decrease with hibernation duration of the host? (3) Do parasitic mites leave the host at the beginning of hibernation? (4) Can parasitic mites and/or their eggs survive the winter (7 months) in the soil?

MATERIALS AND METHODS

Study species

The mite *Riccardoella limacum* is a monoxenous parasite that lives in the mantle cavity of land snails (Fain and van Goethem, 1986). The total life cycle of *R. limacum* lasts 19–23 days at temperatures of 20–25 °C. Female mites lay their eggs in the respiratory tissue of the host. After 3 nymphal stages, mites develop to sexually mature males and females of 0·4 mm body size (Baker, 1970*b*).

Arianta arbustorum is a simultaneously hermaphroditic land snail common in moist habitats of north-western and central Europe, living at elevations of up to 2700 m above sea level (Kerney and Cameron, 1979). The snail has determinate growth (shell breadth of adults 17–24 mm; Baur, 1984). Individuals become sexually mature at 2–4 years old and adults live for another 3–4 years (Baur and Raboud, 1988). *Arianta arbustorum* mates repeatedly in the course of a reproductive season (Baur, 1988).

Experiment 1: Host survival and intensity of infection during hibernation

We randomly collected 240 adult individuals of *A. arbustorum* in a parasite-free population on an alpine pasture with scattered scree, near Gantrisch, 20 km south of Bern, Switzerland (46°42′19″N, 7°26′42″E; altitude 1810 m) on 29 August 2010. The snails were kept individually in transparent plastic beakers (6·5 cm in diameter, 8 cm deep) on moist soil mixed with powdered limestone under a light/dark cycle of 16:8 h and a constant temperature of 19 °C. The soil was obtained from the snails' site of origin. The beakers were cleaned 1–2 times per week and a diet of fresh lettuce was provided *ad libitum*.

After 4 weeks, we experimentally infected half of the snails with *R. limacum* (the other half served as controls). Mites were obtained from parasite-infected *A. arbustorum* collected near Nuglar, 15 km south–east of Basel (47°28′42″N, 7°41′38″E; altitude 434 m). We transferred 6 adult mites to the soft body of an uninfected snail using a small, moistened paintbrush. When the transmitted mites had entered the pulmonary cavity, we put the newly infected snail back into its beaker (Schüpbach and Baur, 2010*a*). Mite intensity of experimentally infected snails was in the range of infection intensities recorded in natural populations (0·3–8·0 mites/snail; Baur and Baur, 2005). Transmitted mites were allowed to reproduce for 6 weeks on their host i.e. 2 mite generations under the conditions described above (Graham *et al*. 1996). We measured the size of each snail (shell diameter) to the nearest 0·1 mm using vernier callipers.

Infected snails and those of the control group were randomly assigned to all 4 groups of about 30 individuals that were allowed to hibernate for 4, 5, 6 or 7 months. Hibernation occurred at 4 °C, in darkness, in beakers lined with 4 cm of moist soil. These conditions are similar to those of hibernating snails buried in the field (Baur, 1994). Hibernating snails (infected and uninfected) were checked every second week to record dead animals. After a hibernation period of 4, 5, 6 or 7 months, infected and uninfected snails were kept again under the same conditions as in the pre-hibernation period for 3 weeks (see above). Infected and uninfected snails did not differ in shell size at the beginning of the experiment (ANOVA, $F_{1,232}=0{\cdot}552$, $P=0{\cdot}458$) and individuals assigned to the 4 groups with different hibernation duration did not differ in shell size (ANOVA, $F_{3,230}=0{\cdot}027$, $P=0{\cdot}994$).

We assessed the parasite load (= intensity of infection) of infected and control snails immediately prior to hibernation and in the 3-week period after hibernation using a non-invasive screening method (Baur and Baur, 2005). The soft body and respiratory pore of each individual were carefully examined for mites using a binocular microscope. The intensity of parasites was expressed as the mean number of mites observed in 3 examinations on 3 different days (Baur and Baur, 2005). A snail was considered as infected if at least 1 mite could be detected during the 3 observations.

Three weeks after hibernation, the hosts were frozen for a posterior screening of mites in the lung

tissue. Snails were fixed in 70% ethanol and dissected under a binocular microscope (Leica MZ8) to quantify the number of mites and eggs in the lung. To assess the potential effect of parasitic mites on the reproductive system of the host, we determined the dry mass of the albumen gland of each snail.

Experiment 2: Short-term response of parasitic mites

To examine whether parasitic mites leave the host at the beginning of the hibernation, we collected 81 adult individuals of *A. arbustorum* from a naturally infected population near Nuglar (47°28′42″N, 7°41′38″E; altitude 434 m) on 18 September 2010. The snails were kept individually in beakers (depth 8 cm, diameter 6·5 cm) lined with moist soil at 19 °C and a 16:8 light/dark cycle. The soil used was from the site Gantrisch, where no mites occur. We repeated the non-invasive parasite screening 5 times. After 19 days, 45 infected snails were randomly assigned to 7 groups each consisting of 6–8 individuals. The groups did not differ in mean shell size ($F_{6,38}=0{\cdot}016$; $P=0{\cdot}999$) and parasite load ($F_{6,38}=1{\cdot}541$; $P=0{\cdot}191$). The snails were allowed to hibernate as described above for periods of 0 (control), 4, 8, 12, 24, 48 or 72 days. After hibernation, snails of the corresponding group were frozen and the actual number of mites and eggs in the lung tissue was determined for each host as described above.

Experiment 3: Mite survival in the soil

To examine whether parasitic mites survive in the soil during the hibernation period of snails, beakers containing soil on which experimentally infected snails from Gantrisch ($n=34$), natural infected snails from Nuglar ($n=26$) or uninfected snails from Gantrisch ($n=26$, control) had been maintained singly for 6 weeks, were transferred to hibernation conditions (4 °C, darkness), but without snails. After a period of 7 months, uninfected individuals of *A. arbustorum* from Gantrisch were placed singly in the beakers with the 3 soil treatments and kept under standard conditions (19 °C, 16:8 dark/light cycle; as described above) for 3 months. After 3 months, snails were frozen and a posterior screening of mites and their eggs in the lung tissue was performed as described above.

Statistical analysis

Data analysis was performed using R Statistical Software (R Development Core Team, 2011). We used Generalized Linear Models (GLM) with binomial distribution to examine the effects of parasite infection (categorical predictor), hibernation duration and size of individual snails (both continuous predictors) on host survival (Exp. 1). The minimal adequate model was selected by Akaike Information Criterion (AIC). We used linear models (LM) to test whether the intensity of mite infection changed with increasing duration of hibernation and with snail size as well as to examine whether the dry weight of the albumin gland (log (x + 1)-transformed) differed between infected and uninfected snails and was affected by hibernation duration and snail size. We used GLM with Poisson distribution to examine whether the numbers of mites and eggs found in the lung tissue of hosts were influenced by hibernation duration (Exp. 2).

RESULTS

Experiment 1: Host survival and intensity of infection during hibernation

Host survival was affected negatively by parasite infection but not by the duration of hibernation and the size of individual snails (Fig. 1). Statistical model selection removed the factors hibernation duration and shell size as well as the two-way interactions keeping only the factor parasite infection in the minimal adequate model (GLM, estimate = −0·678, S.E. = 0·332, $z=-2{\cdot}044$, $P=0{\cdot}041$). Overall, 80 out of the 108 infected snails (74·1%) survived hibernation as did 107 of the 126 (84·9%) uninfected snails. The intensity of infection prior to hibernation averaged 2·9 mites (range 0·3–12·0, $n=108$). Survival of host snails during hibernation tended to be negatively affected by the intensity of infection assessed prior to hibernation (GLM, estimate = −0·783, S.E. = 0·425, $z=-1{\cdot}841$, $P=0{\cdot}062$).

The intensity of infection decreased with increasing hibernation duration (Fig. 2). Model selection removed the factor snail size and the two-way interaction keeping only the factor hibernation duration in the minimal adequate model (LM, estimate = −0·500, S.E. = 0·186, $t=2{\cdot}688$, $P=0{\cdot}008$). The decrease in parasite load, expressed by the difference between intensity of infection prior to hibernation and intensity of infection after hibernation, was 1·5 ± 0·4 mites after 4 months ($n=22$), mean ± S.E., 2·0 ± 0·3 mites after 5 months ($n=19$), 2·3 ± 0·4 mites after 6 months ($n=20$), and 3·1 ± 0·6 mites after 7 months ($n=19$).

The dry weight of the albumen gland, an estimate of the female reproductive capacity in the successive season, did not differ between infected and parasite-free host snails (LM, estimate = 0·028, S.E. = 0·218, $t=0{\cdot}129$, $P=0{\cdot}898$). Furthermore, the dry weight of the albumen gland was neither affected by hibernation duration (estimate = −0·068, S.E. = 0·075, $t=0{\cdot}911$, $P=0{\cdot}365$) nor by snail size (LM, estimate = −0·018, S.E. = 0·116, $t=0{\cdot}158$, $P=0{\cdot}875$).

Considering exclusively infected host snails that survived, the detailed examination of the lung tissue 3 weeks after hibernation revealed a mean number

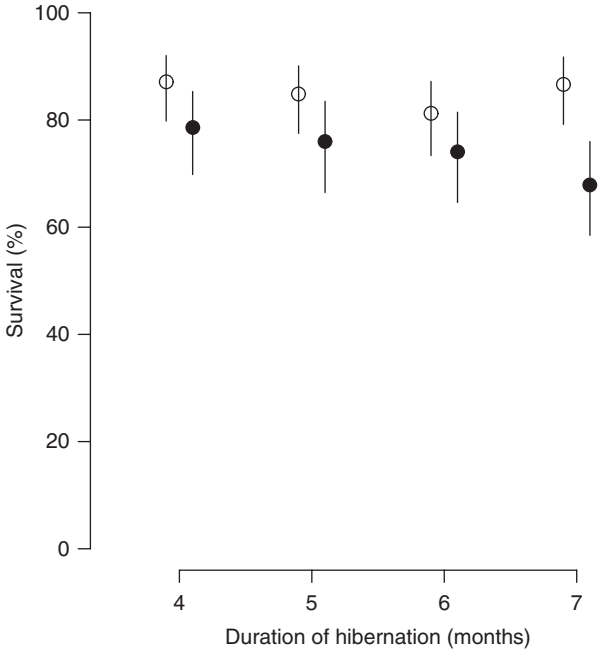

Fig. 1. Survival of infected (black dots) and uninfected (open dots) host snails in relation to hibernation duration. Whiskers represent standard errors based on binomial distributions. Sample sizes for infected snails were 28, 25, 27 and 28, those for uninfected snails were 32, 33, 32 and 30.

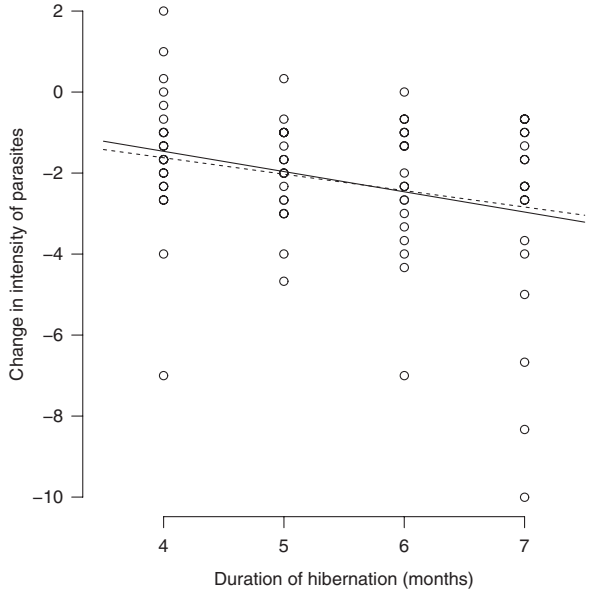

Fig. 2. Decrease in intensity of parasitic mites in relation to hibernation duration of the host snail *Arianta arbustorum*. Open dots represent single snails. The solid line represents the fitted linear model ($Y=-0{\cdot}500X+0{\cdot}538$; $R^2=0{\cdot}085$, $n=80$, $P=0{\cdot}008$), the dashed line the linear model including the zero point (no intercept: $Y=-0{\cdot}405X$; $R^2=0{\cdot}596$, $n=80$, $P<0{\cdot}001$).

of 10·2 mites (S.E. = 2·0, range: 0–77, $n=80$) and of 8·3 eggs (S.E. = 1·8, range: 0–87, $n=80$). The number of mites found in the lung was negatively affected by the hibernation duration of the host (GLM, estimate = −0·101, S.E. = 0·031, $z=-3{\cdot}224$, $P=0{\cdot}001$). However, the number of mite eggs was not influenced by hibernation duration (GLM, estimate = −0·035, S.E. = 0·034, $z=-1{\cdot}010$, $P=0{\cdot}312$).

Experiment 2: Short-term response of parasitic mites

Host snails kept under hibernation conditions for 0 (control), 4, 8, or 12 days did not differ in the number of mites and mite eggs found in the lung tissue (mites: ANOVA, $F_{3,20}=1{\cdot}173$, $P=0{\cdot}345$; eggs: $F_{3,20}=0{\cdot}054$, $P=0{\cdot}983$). This indicates that none or very few mites left their hosts at the beginning of hibernation. Consequently, data of host snails kept under hibernation conditions for 0–12 days were combined for further analyses. Host snails hibernating for different periods showed different numbers of mites (Fig. 3; ANOVA, $F_{3,41}=2{\cdot}854$, $P=0{\cdot}048$). In contrast, the number of mite eggs found in the lung tissue did not change with increasing hibernation duration of the host snails (Fig. 3; $F_{3,41}=1{\cdot}206$, $P=0{\cdot}319$).

Experiment 3: Mite survival in the soil

No parasitic mites and eggs were found in the lung tissue of originally parasite-free snails ($n=26$) kept on control soil for 6 weeks. Similarly, no parasitic mites and eggs were recorded in originally parasite-free snails ($n=26$) kept on soil on which naturally infected snails from the population Nuglar had previously been maintained before the soil was stored at 4 °C for 7 months. However, 1 single mite egg but no adult mite was found in the lung tissue of one of the 34 originally parasite-free snails kept on soil on which experimentally infected snails from the population Gantrisch had previously been maintained before it was stored at 4 °C for 7 months. This indicates that mite survival in the soil might be very low during winter.

DISCUSSION

Our study provides experimental evidence that winter survival of the host snail *A. arbustorum* is negatively affected by *R. limacum*, but not by the size of the snails. Furthermore, the intensity of mite infection decreased with increasing hibernation duration of the host. Reduced winter survival has also been found in naturally infected individuals of *A. arbustorum* in 2 out of 3 populations (Schüpbach and Baur, 2008*a*). In experimentally infected *A. arbustorum*, mortality during hibernation was both influenced by the intensity of infection and family origin (Schüpbach and Baur, 2010*a*). Similarly, in the present study, host survival during the winter tended to be negatively affected by the intensity of infection.

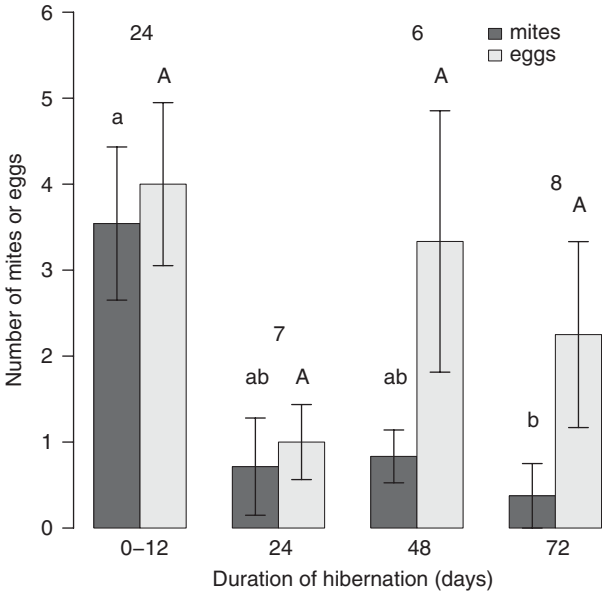

Fig. 3. Number of mites and eggs found in the lung tissue of the host snail *Arianta arbustorum* after hibernation periods of different lengths. Means ± 1 S.E. are shown with sample size on the top of bars. Small letters (in mites) and capitals (in eggs) indicate significant differences between hibernation periods of different lengths.

Differences in winter survival of parasitized and parasite-free hosts may directly affect gene frequencies in the host population and thus influence the host-parasite co-evolution (Mitchell *et al*. 2005).

Any estimate of intensity of infection relies on the accuracy of the method used for counting the parasites. In our study organism, the non-invasive estimate could be influenced by uncertainties in the recognition of lightly infected animals. The dissection of snails with a careful examination of their lung epithelium is the most precise but also time-consuming method, provided that the mites do not leave the freshly-killed host. We did not observe that mites left dead *A. arbustorum*, which were killed by freezing. However, apart from the ethical issue of killing animals, potential effects of parasite infection on the hosts can no longer be examined. These problems can be avoided by using non-invasive parasite screening methods. The non-invasive method used in this study is fairly accurate and its precision can further be improved by increasing the number of visual mite assessments (e.g. from 3 to 5 times, as done in Exp. 2), which is feasible in snails kept in controlled laboratory experiments (Baur and Baur, 2005).

Riccardoella limacum depends on its host to complete the life cycle (Baker, 1970*b*). Food uptake and reproduction are only possible in the mantle cavity of the host (Baker, 1970*a*). Mites are frequently transmitted during courtship and mating of *A. arbustorum*. The parasites move over the soft body of the snail and switch to the new host. Furthermore, *R. limacum* has been found to be successfully transmitted via soil without physical contact (Schüpbach and Baur, 2008*b*). Experiments showed that the blind mites use fresh mucus trails of snails to locate new hosts (Schüpbach and Baur, 2008*b*). In this way they also infect juvenile snails. Baker (1970*a*) studied the off-host survival of *R. limacum* and found that mites kept in culture cells survived for 8 days without food supply at room temperature. In moist soil kept at 19 °C, individuals of *R. limacum* survived and infected new snails (Schüpbach and Baur, 2008*b*). In the present study, none or only very few mites survived in moist soil kept at 4 °C for 7 months. We found only a single mite egg in 1 out of 60 originally parasite-free snails maintained on these soils. This indicates that 1 mite may have survived in the soil before it could find a new host and deposit the egg in its lung.

The influence of a parasite on its host may change in response to altered physical conditions of the environments. When infected gastropods aestivate, such as during summer droughts, parasite development is normally inhibited (Solomon *et al*. 1996) and host survival is reduced compared to uninfected snails. Temperature can be a limiting factor on parasite development, both within invertebrate hosts and in free-living parasites. At low temperature, larval development of the nematode *Elaphostrongylus rangiferi* (Mitskevich 1958) was delayed in the host snails *A. arbustorum* and *Euconulus fulvus* (Müller 1774) and 1st-stage larvae of this lung worm showed a higher survival than their successive stages in hibernating hosts (Halvorsen and Skorping, 1982; Schjetlein and Skorping, 1995). The minimum temperature necessary for the development of *R. limacum* is not known. However, the number of mites found in the lung tissue was reduced in hosts hibernating for 24 days or longer, while the number of eggs did not change with increasing hibernation duration. In Experiment 2, which had shorter recording intervals, mites did not leave the host when the snails buried in the soil at the beginning of hibernation. However, the number of mites found in the lung tissue decreased when the hosts hibernated for 24 days or longer, while the number of eggs did not change with increasing hibernation duration. Thus, most parasitic mites died and no eggs hatched during hibernation. We frequently observed clumps of white mucus containing dead mites in the respiratory pore of parasitized snails. This could represent a kind of lung cleaning in infected hosts. In spring, when the temperature increases and the host snails emerge from hibernation, the small parasite population might mainly stem from surviving eggs. During the host's activity season the mite population increases, reaching a peak in autumn. This explanation is in line with the seasonal pattern in mite number recorded in *A. arbustorum* and *C. aspersum* (Graham *et al*. 1996; Baur and Baur, 2005). The seasonal increase in number of mites might depend

on the temperature and the length of the snail's activity season.

The prevalence of infection by *R. limacum* in populations of *A. arbustorum* is highly variable. In Switzerland, infected populations occurred at localities with a higher mean temperature in June (a surrogate for the length of the vegetation period) than populations without mites (Baur and Baur, 2005). Parasitic mites did not occur in snail populations situated at altitudes of 1290 m or higher. Climatic factors are known to have a significant effect on the occurrence of helminth parasites in terrestrial gastropods. For example, Morley and Lewis (2008) found a positive correlation between winter maximum temperature and total sporocyst prevalence of trematodes *Dicrocoelium* sp. in a land snail community in southern England.

Snails are ectothermic animals and are particularly vulnerable to climatic change, not only due to physiological responses but also to a shift in plant community composition and structure impacting on their population dynamics (Baur and Baur, 1993*b*; Sternberg, 2000). In the temperate zone, global climate change will probably lead to shorter and milder winters, resulting in shorter periods of hibernation. Climate warming may extend the active season of terrestrial gastropods and increase the availability of free-living parasites (Harvell *et al.* 2002). In the host *A. arbustorum*, hibernation duration may decrease and winter survival of *R. limacum* will increase. The extended active season of the host may allow the parasitic mite to spread to higher altitudes by dispersing snails (Baur and Baur, 1993*a*).

ACKNOWLEDGEMENTS

We thank Anette Baur and an anonymous referee for valuable comments on the manuscript. Financial support was received from the Swiss National Foundation.

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Chapter II

Parasitic mites influence intra- and interpopulational variation in sperm length in a simultaneous hermaphrodite land snail (Gastropoda: Helicidae)

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Biological Journal of the Linnean Society, 2014, **113**(4): 1036–1046

The Linnean Society of London
BIOLOGICAL Journal of the Linnean Society

Biological Journal of the Linnean Society, 2014, **113**, 1036–1046. With 5 figures

Parasitic mites influence intra- and interpopulational variation in sperm length in a simultaneous hermaphrodite land snail (Gastropoda: Helicidae)

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Received 29 April 2014; revised 1 July 2014; accepted for publication 1 July 2014

Sperm morphology can be highly variable among species, but less is known about patterns of population differentiation within species. Sperm morphology is under strong sexual selection, may evolve rapidly, and often co-varies with other reproductive traits that differ between populations. We investigated variation in sperm morphology in the simultaneous hermaphrodite land snail *Arianta arbustorum* in relation to parasitic mite infection. Variation in total sperm length and sperm head length was assessed in 23 populations sampled across the distributional range of the species in Central and Northern Europe. We found a pronounced variation in total sperm length among the populations studied, with a difference of 11.0% of total sperm length between the shortest and longest population means. Differences among populations explained 62.9% of the variance in total sperm length, differences among individual snails within population 23.4% and differences within individual snail 13.7%. Mantel tests showed that interpopulation differences in total sperm length increased significantly with geographical distance between populations. A minimal adequate model revealed that parasitic infection had a positive effect and longitude a negative effect on total sperm length. Thus, independent of the population examined, mite-infected individuals of *A. arbustorum* produced larger sperm than uninfected snails and total sperm length decreased from west to east. Sperm head length also varied among populations, but it was not influenced by any of the factors examined. In a subsample of 12 populations restricted to the mountains of Switzerland (elevational range 440–2485 m a.s.l.), total sperm length decreased with increasing elevation. Our results suggest that selection pressures acting among populations may differ from those acting within. Stabilizing selection might be a possible mechanism for producing the reduced variation observed in sperm length within a population.

ADDITIONAL KEYWORDS: *Arianta arbustorum* – elevation – geographical variation – parasite – sperm length.

INTRODUCTION

Sperm show an extraordinary variation in size and shape both within and among species (Snook, 2005; Manier & Palumbi, 2008; Pitnick, Hosken & Birkhead, 2009). This variation may reflect population- and species-specific differences in fertilization mode, allometry and strength of post-copulatory sexual selection. Within species, sperm morphology is assumed to be maintained by stabilizing selection (Parker, 1993; Calhim, Immler & Birkhead, 2007), but it can respond to directional selection, including selection pressure from the female reproductive tract (e.g., Morrow & Gage, 2001; Miller & Pitnick, 2002). Hence, if spatially segregated populations vary in strength or form of selection, sperm might be selected towards different optima across populations.

Despite great interest in evolutionary diversification of sperm morphology and its implication on reproductive success, information on geographical variation in sperm length is very limited. Pitnick *et al*. (2003) found highly significant differences in

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sperm length among eight populations distributed throughout the geographic range of the fruit fly *Drosophila mojavensis* endemic to the Sonoran Desert. In the red-winged blackbird *Agelaius phoeniceus*, sperm length gradually increased from southwest to northeast of the breeding range in North America (Lüpold, Westneat & Birkhead, 2011). Significant differences in sperm length were also reported for four populations of the quacking frog *Crinia georgiana* (Hettyey & Roberts, 2006). Some of the geographical variation in sperm length is likely attributable to differences in the duration of the breeding season, which is shorter both at high latitude and high elevation than at low latitude and low elevation. There is some experimental evidence that temperature can influence sperm length in invertebrates (e.g., in yellow dung flies *Scathophaga stercoraria*, Hellriegel & Blanckenhorn, 2002).

Parasites often have deleterious effects on the reproductive success of their hosts. Parasite coevolution and its consequences for sexual selection have been intensively investigated (reviewed by Agrawal, 2006). Parasites may alter both the mating behaviour and mating frequency of male hosts (Moore, 2002). Few studies, however, have attempted to examine parasite influences on ejaculate qualities and sperm length. In arthropods, the intracellular microbe *Wolbachia* affects sperm during spermatogenesis causing 'cytoplasmic incompatibility' (Karr, Swanson & Snook, 2009). Infected males mate normally, transfer sperm and sperm fertilize eggs, but the eggs fail to complete karyogamy and die. In males of the beetle *Tribolium castaneum*, sperm precedence was significantly decreased by tapeworm infection (Yan & Stevens, 1995).

Gastropods exhibit a large interspecific variation in sperm morphology (Thompson, 1973; Healy, 1988, 1996; Hodgson *et al*., 1996). Spermatozoa of stylommatophorans are among the largest of the gastropods (e.g., 850 μm in *Helix pomatia* and 1140–1400 μm in *Hedleyella falconeri*; Thompson, 1973). Information on intraspecific variation is restricted to a single species, the land snail *Arianta arbustorum* (Linneaus 1758), with mean sperm length of 878, 898, 913 and 939 μm in four populations (Minoretti & Baur, 2006). Sperm are monomorphic in this snail. So far, the significance of the variation in sperm length in terrestrial gastropods is largely unknown.

The present study focuses on intraspecific variation in sperm length in the simultaneously hermaphroditic land snail *A. arbustorum*. The species is widespread in Central and Northwestern Europe (Kerney & Cameron, 1979) and shows a high phenotypic variability in shell and soft body colour that is related to habitat and elevation (Burla & Stahel, 1983). *Arianta arbustorum* exhibits large interpopulational genetic variation (Arter, 1990; Haase *et al*., 2003) and shows considerable variation in behaviour (Baur, 1986, 1994a; Baur & Gosteli, 1986) and rate of parasitic infestation (Baur & Baur, 2005). There is experimental evidence for sperm competition in this species: multiple mating is common (Baur, 1994b), viable sperm from different males can be stored for long periods (Baur, 1988), and multiple paternity and differential male fertilization success have been recorded (Kupfernagel, Rusterholz & Baur, 2010; Kupfernagel & Baur, 2011). The snail's female role has some control over the fertilization of the eggs by selective sperm use (Baur, 1994b; Bojat & Haase, 2002).

A fundamental assumption of sex allocation theory in simultaneous hermaphrodites is a trade-off between female and male functions, i.e. the animal has a fixed amount of resources to allocate between the genders (Charnov, 1982). A hermaphrodite that suppresses the use of one sex function may automatically free resources for the other sex function. Parasite infection reduces egg production in snails (Schüpbach & Baur, 2008a; see below). In this case, classical sex allocation theory predicts an increased male allocation by producing more or larger sperm (Charnov, 1982, 1996).

The aim of the present study was to examine total sperm length and sperm head length across the distribution range of *A. arbustorum* to answer the following questions: (1) Do total sperm and sperm head lengths vary across populations? (2) Do site characteristics of the populations sampled (latitudinal, longitudinal, elevation) contribute to the variation in total sperm and sperm head lengths? and (3) Do individual snail traits such as parasite infection and shell size influence total sperm length and sperm head length?

MATERIAL AND METHODS

Study organism

Arianta arbustorum (Pulmonata, Helicidae) is common in moist habitats of Northwestern and Central Europe reaching elevations of up to 2700 m (Kerney & Cameron, 1979). The snail has determinate growth (shell breadth of adults 16–22 mm). Individuals become sexually mature at 2–4 years, and adults live another 3–4 years (maximum 14 years; Baur & Raboud, 1988). Individuals mate repeatedly with different partners in the course of a reproductive season and are able to store fertile sperm for more than 1 year (Baur, 1988, 2007). In the field, snails deposit one to three egg batches consisting of 20–50 eggs, per reproductive season (Baur, 1990).

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 transfers simultaneously one spermatophore (Haase & Baur, 1995). The spermatophore is formed and filled with sperm during copulation. 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, Locher & Baur, 1998). Snails need at least 8 days to replenish their sperm reserves after a successful copulation (Locher & Baur, 1999; Hänggi, Locher & Baur, 2002). Mating is random with respect to shell size and different degrees of relatedness (Baur, 1992; Baur & Baur, 1997). Outcrossing is the dominant mode of reproduction, but self-fertilization may occur in low frequencies, particularly after long periods of isolation (Chen, 1994).

Individuals of *A. arbustorum* can be infected by the ectoparasite mite *Riccardoella limacum* (Schrank 1776) (Baur & Baur, 2005). The mite feeds on blood in the mantle cavity of its host (Baker, 1970). Parasite infection reduces the activity of snails and their egg production, and decreases winter survival (Schüpbach & Baur, 2008a). The mites are transmitted through physical contacts of the hosts (Baur & Baur, 2005), via mucous trails of the host snail or indirectly via soil (Schüpbach & Baur, 2008b). Natural snail populations vary widely in prevalence of mite infection (0–78% in 11 populations; Baur & Baur, 2005).

Sample collection

We analyzed the sperm of 255 *A. arbustorum* from 23 locations (hereafter referred to as populations) situated at elevations ranging from 1 to 2485 m above sea level throughout northern and central Europe (see study locations in Table 1). The sampling sites cover a major part of the species' distribution area. At each location, we collected 12 snails (for exceptions see Table 1) within an area of 25 m × 25 m and transported them to the laboratory, where they were immediately frozen at –80 °C. We recorded the geographical coordinates and elevation of each location using a GPS receiver (Garmin, Geko 201, Romsey, U.K.) and assessed the habitat type (Table 1).

Sperm measurements

In helicid snails, the autosperm produced are stored in the mid-section of the hermaphrodite duct, the vesicula seminalis (Hodgson, 1996). We dissected out the hermaphrodite duct of each individual. Using a razor blade, we opened the wall of the sperm-containing part of the hermaphrodite duct along its longitudinal axis. To release spermatozoa we placed the opened hermaphrodite duct for 12 h in 500 μl PBS (pH 7.2) at 4 °C. The sperm suspension was used for sperm measurements following the method of Minoretti & Baur (2006). In brief, we placed aliquots of 20 μl sperm suspension on two microscopic slides. The samples were covered with a coverslip and sealed with nail polish. We digitized randomly chosen spermatozoa using a camera (Canon PowerShot S70) mounted on a compound microscope (Leica DMLD, PH3, ×100 objective lens) connected to a Macintosh computer. From these images, we measured total sperm length (head and tail) and sperm head length for 25 spermatozoa from each snail using ImageJ (version 1.43f).

We assessed the reliability of multiple length measurements (N = 8 measurements on 8 different days) on the same sperm (N = 12 spermatozoa) by calculating the repeatability following Lessells & Boag (1987). Repeatabilities of measurement were 0.99 for total sperm length and 0.80 for sperm head length, indicating that the technique was highly accurate for total sperm length but less accurate for head length. The latter can be explained by the fact that the distinction between head, midpiece, and tail is difficult to see in helicid spermatozoa (Dohmen, 1983). Broken sperm tails could bias our measurement of total sperm length. In the case of a large deviation of a particular sperm length from the average value of all sperm of the individual, we excluded this spermatozoon from the analyses. In total, 6324 sperm from 255 individuals collected in 23 populations were analyzed.

To examine the prevalence of parasitic mite infection, we dissected the snails and carefully checked their lung epithelium for the presence of mites and/or their eggs using a binocular microscope (for method see Haeussler *et al.*, 2012). To control for snail size, we measured the shell width of each individual to the nearest 0.1 mm using vernier callipers (the shell width of one individual could not be measured). Shell width is a more reliable measurement of snail size than mass, because mass depends on the state of hydration and thus is highly variable in terrestrial gastropods (Baur, 1992). Obtained data are available in Haeussler, Schmera & Baur (2014).

Statistical analyses

Data analyses were performed in the R statistical environment (R Development Core Team, 2012). We used mixed-effect models (with individual snail as random factor) to test whether total sperm length and sperm head length differed among the populations examined (GLMM in the *lme4* package; Bates, Maechler & Bolker, 2013). Variance component analysis was performed to quantify the extent of variation in total sperm length and sperm head length among and within populations and within individuals in each population. Following Crawley (2007) we fitted a

Table 1. Locations of sampled populations, elevation, vegetation of the habitat and sampling date together with sample sizes (number of snails and number of sperm used in the data analyses)

ID	Localities	Geographical coordinates	Elevation (m a.s.l.)	Habitat type	Number of snails*	Sampling date
1	Trondheim, Norway	63° 25′ 39.26″ N 10° 21′ 30.02″ E	72	Forest	12	25.07.2012
2	Edsåsdalen, Sweden	63° 18′ 52.33″ N 13° 04′ 59.60″ E	545	Rough herbage	12	22.07.2012
3	Uppsala, Sweden	59° 50′ 45.97″ N 17° 37′ 03.49″ E	38	Rough herbage	12	29.07.2012
4	Delsjö, Gothenburg, Sweden	57° 41′ 33.16″ N 12° 01′ 09.19″ E	75	Rough herbage	12	14.07.2011
5	Emmaboda, Sweden	56° 37′ 50.08″ N 15° 33′ 23.75″ E	127	Forest	12	31.07.2012
6	Danzig, Poland	54° 24′ 07.40″ N 18° 33′ 39.68″ E	49	Rough herbage	12†	25.06.2012
7	Bremen, Germany	53° 10′ 16.70″ N 8° 38′ 51.86″ E	19	Rough herbage	12	11.07.2012
8	Amsterdam, the Netherlands	52° 18′ 20.99″ N 4° 50′ 35.98″ E	1	Rough herbage	7	20.06.2011
9	Luppe, Kleinliebenau, Germany	51° 22′ 34.42″ N 12° 12′ 56.62″ E	100	Rough herbage	11	29.08.2011
10	Viersen, Germany	51° 15′ 26.06″ N 6° 26′ 12.29″ E	37	Rough herbage	10	15.07.2012
11	Bebenhausen, Schönbuch, Germany	48° 34′ 10.82″ N 9° 02′ 01.33″ E	415	Forest	12	09.07.2011
12	Nuglar, Switzerland	47° 28′ 41.85″ N 7° 41′ 37.77″ E	440	Rough herbage	12	29.08.2010
13	Mont Raimeux, Switzerland	47° 18′ 26.68″ N 7° 25′ 45.64″ E	1290	Forest	12	24.07.2011
14	Corgémont, Switzerland	47° 09′ 57.52″ N 7° 08′ 12.09″ E	1155	Forest	9	06.08.2011
15	Les Roches, Switzerland	47° 08′ 14.01″ N 7° 04′ 23.53″ E	1515	Alpine grassland	12‡	06.08.2011
16	Pilatus, Switzerland	46° 58′ 17.03″ N 8° 15′ 42.95″ E	1577	Alpine grassland	9	11.08.2012
17	Linthal, Switzerland	46° 55′ 43.30″ N 8° 59′ 57.56″ E	680	Forest	12	09.06.2012
18	Scuol, Switzerland	46° 48′ 57.66″ N 10° 14′ 18.64″ E	2485	Alpine grassland	12	26.08.2012
19	Sörenberg, Switzerland	46° 48′ 13.49″ N 8° 04′ 05.30″ E	1360	Alpine grassland	9	02.10.2011
20	Gurnigel, Switzerland	46° 45′ 08.06″ N 7° 27′ 09.63″ E	1330	Forest	12	09.05.2009
21	Gantrisch, Switzerland	46° 42′ 18.70″ N 7° 26′ 42.10″ E	1800	Alpine grassland	12	22.05.2011
22	Vallatscha d'Astras, Switzerland	46° 39′ 55.29″ N 10° 20′ 15.15″ E	2270	Alpine grassland	11§	25.08.2012
23	Müstair, Switzerland	46° 37′ 52.62″ N 10° 27′ 32.41″ E	1228	Rough herbage	9¶	24.06.2011

*Number of snails examined. For each snail 25 sperm were measured.
†11 snails: 25 sperm; 1 snail: 11 sperm.
‡10 snails: 25 sperm; 2 snails: 20 sperm.
§10 snails: 25 sperm; 1 snail: 6 sperm.
¶8 snails: 25 sperm; 1 snail: 17 sperm.

Table 2. Summary of ANOVA table for mixed-effect models examining the effect of population on total sperm length and sperm head length

Response variable	Predictor	numDF	denDF	F	*P*
Total sperm length	Population	22	232	31.4	< 0.001
Sperm head length	Population	22	232	13.5	< 0.001

Individual snail is a random factor.
Number of individuals = 255.
Number of sperm measurements = 6324.

mixed-effect model with two hierarchically ordered random factors (population and individual nested in population) and examined the variance of the statistical model.

To analyze the spatial variation in total sperm length and sperm head length, we transferred longitude and latitude of all sampling sites into Cartesian coordinates and constructed a distance matrix for the 23 geographical locations using the *fossil* package (Vavrek, 2011). The pairwise geographic distances among the 23 sampling sites ranged from 5.2 to 1922.0 km (mean ± SE = 831.5 ± 37.5 km, $N = 253$). We performed Mantel tests with 999 permutations using the *vegan* package (Oksanen *et al*., 2013) to establish whether the interpopulational differences in sperm length and sperm head length increased with the geographical distances among populations. We calculated Mantel correlograms using the *ecodist* package (Goslee & Urban, 2007) to characterize the spatial patterns of differences both in total sperm length and sperm head length among populations.

For each location, data on mean annual temperature and annual precipitation were obtained from nearby situated weather stations, from local climatic atlases or by using the mean surface climate data per cell on a 0.25 degree grid from the E-OBS dataset via the Climate Explorer web-site (http://climexp.knmi.nl).

We applied Pearson correlation to test the interdependency of the predictors latitude, longitude, elevation, mean annual temperature and annual precipitation at the population level ($N = 23$ in each case). Elevation and latitude were highly intercorrelated, as were latitude with annual precipitation, elevation with annual precipitation and elevation with mean annual temperature (see Supporting Information, Table S1). Other predictors were less strongly intercorrelated (see Supporting Information, Table S1). Thus, populations situated at high elevation were exposed to lower mean annual temperature, larger annual precipitation and occurred in the southern part of the species distribution area (in the Alps) than populations situated at low elevation, which were exposed to higher mean annual temperature and lower annual precipitation. We therefore only used the predictors elevation and longitude in the further analyses keeping in mind that elevation represents also latitude, mean annual temperature and annual precipitation.

At the level of individuals ($N = 255$), each snail was characterized by its shell width and the presence or absence of parasite mites. We used mixed-effects models with population as the random factor in the *nlme* (Pinheiro, Bates & DebRoy, 2013) and *lme4* packages (Bates *et al*., 2013) to incorporate any possible dependency of individual characters on predictors at the population level. The same type of model was used to examine the dependency of individual characters on elevation and longitude. We also applied mixed-effects models with population and snail identity nested in population to examine the effects of elevation, longitude, shell width and parasite infection on total sperm length and sperm head length. Model selection was performed using Akaike Information Criteria (AIC).

Eleven of the 23 populations sampled are located in lowlands, while 12 populations are from the mountains in Switzerland covering an elevational range from 440 to 2485 m a.s.l. (no. 12–23 in Table 1; see Supporting Information, Table S1). We used a reduced data set consisting of the 12 mountain populations to examine whether the pronounced elevational range and/or any other factors (predictors of mixed-effects models, see above) explain observed total sperm length.

RESULTS

Differences among populations

Total sperm length differed significantly among the 23 populations (Table 2, Fig. 1). Sperm head length differed also among populations (Table 2) with population means ranging from 8.76 to 10.78 μm. Sperm head length and total sperm length were not correlated at the population level ($r = 0.18$, $N = 23$, $P = 0.394$). Variance component analysis revealed that differences among populations explained 62.9% of the variance in total sperm length, differences among individual snails within population 23.4% and

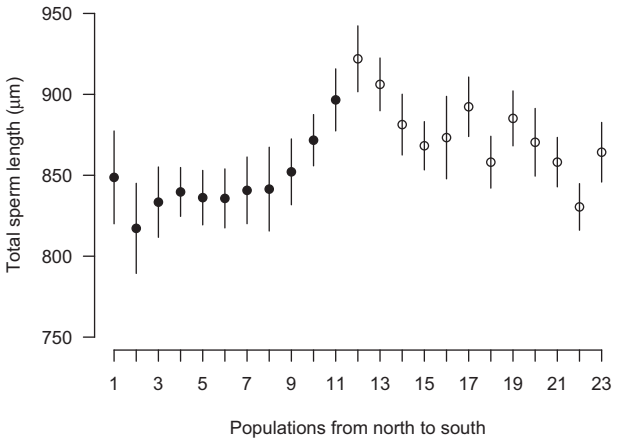

Figure 1. Interpopulation variation in total sperm length (mean ± SD) of *A. arbustorum*. Populations are arranged from north to south and their number codes refer to those presented in Table 1. Open dots indicate the mountain populations in Switzerland.

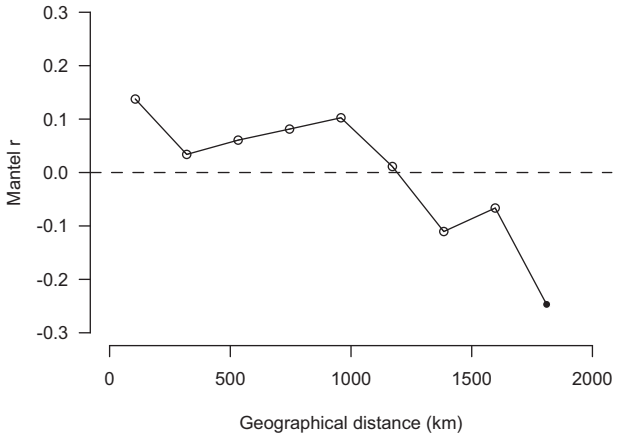

Figure 2. Mantel correlogram showing Mantel r (r_M) in relation to the geographical distances among populations. The full dot refers to r_M significantly different from 0, whereas empty dots indicate r_M-values not significantly different from 0.

differences within individual snail 13.7%. Corresponding values for variance in sperm head length were 18.0%, 11.9% and 70.1%, respectively.

Geographical patterns in sperm size

Mantel tests showed that the interpopulation difference in total sperm length increased significantly with the geographical distance between the populations ($r_M = 0.284$, $P = 0.010$). The Mantel correlogram revealed a typical pattern for a spatial gradient: total sperm length showed positive autocorrelation at short distances and negative autocorrelation at long distances (Fig. 2). In contrast, the Mantel test did not reveal any significant relationship between interpopulation difference in sperm head length and distance between populations ($r_M = -0.074$, $P = 0.661$).

Inter-dependency of variables

Parasitized and non-parasitized snails did not differ in shell width (Table 3). Mixed-effect models revealed that elevation had a negative effect on shell width (Table 3). Thus, the snails' shell width decreased with increasing elevation. Additional tests did not reveal any further significant inter-dependency among predictors at the individual level. Although the mixed-effect model did not show any effect of elevation on parasite infection of individual snails (generalized mixed-effect model, binomial distribution, population as a random factor and elevation as a predictor: estimate = −0.005, s.e. = 0.004, z-value = −1.194, $P = 0.232$; Fig. 3), no infected snail was found in populations situated above an elevation of 1500 m.

Predictors of sperm characters

The minimal adequate model revealed that parasite infection had a positive effect on total sperm length (Table 3, Fig. 4). In contrast, longitude had a negative effect on total sperm length (Table 3). Other factors (elevation, habitat type, shell width) were removed during the model selection procedure. These results indicate that total sperm length of *A. arbustorum* is influenced by parasites and shows a geographical pattern with a decrease from west to east. We also examined the effect of parasite infection on total sperm length considering exclusively populations in which parasite mites occurred (7 populations with 78 individuals and 1936 sperm measurements). The mixed-effect model using population and individual (nested in population) as random factors showed that sperm of infected individuals were longer than those of uninfected individuals (Table 3, Fig. 4).

Examining sperm head length as response variable, the minimal adequate model included longitude and shell width, but neither effect was statistically significant (Table 3). This indicates that sperm head length is not influenced by any of the factors assessed.

Considering data from the 12 mountain populations sampled over an elevational range from 440 to 2485 m a.s.l. (no. 12–23 in Table 1), the minimum adequate model revealed a negative influence of elevation on total sperm length (Table 3). Other factors including shell width were removed during the model selection procedure. Linear regression showed that 67.5% of the variance in sperm length in the mountain populations can be explained by elevation (Fig. 5).

Table 3. Summary of mixed-effect models examining the effects of elevation and presence of parasite on shell width as well as the effects parasite infection, longitude, elevation and shell width on total sperm length and sperm head length

Response variable	Random factor	Predictor	Estimate	s.e.	d.f.	t	*P*
Shell width*	population	Presence of parasite	0.172	0.228	230	0.754	0.452
Shell width*	population	Elevation	−0.001	0.001	21	−2.408	0.025
Total sperm length†	population and individual (nested in population)	Parasite infection	9.177	4.078	230	2.250	0.025
		Longitude	4.489	1.269	21	−3.538	0.002
Total sperm length‡	population and individual (nested in population)	Parasite infection	8.606	4.273	70	2.014	0.048
Total sperm length§	population and individual (nested in population)	Elevation	−0.034	0.006	10	−4.989	< 0.001
Sperm head length†	population and individual (nested in population)	Longitude	−0.043	0.026	21	−1.648	0.114
		Shell width	−0.047	0.026	230	−1.809	0.072

*Number of populations = 23, number of individuals = 254.
†Number of populations = 23, number of individuals = 254, number of sperm measurements = 6299.
‡Number of infected populations = 7, number of individuals = 78, number of sperm measurements = 1936.
§Number of mountain populations = 12, number of individuals = 130, number of sperm measurements = 3213.

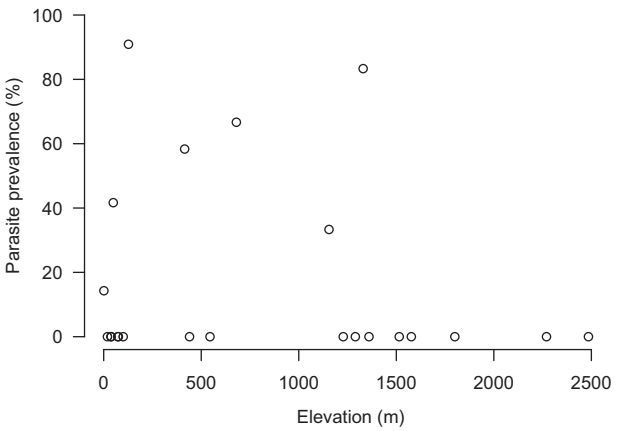

Figure 3. Parasite prevalence (percentage of infected individuals) in relation to the elevation of the populations examined ($N = 23$).

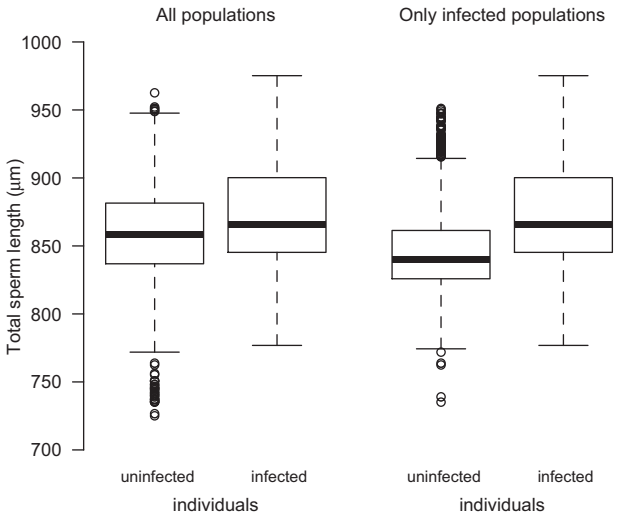

Figure 4. Effect of parasite infection of snails on the total sperm length in all populations (including non-infected populations; left), and only in infected populations (right). The horizontal line indicates the median, the bottom and top of the box the 25th and 75th percentiles, respectively. Whiskers show the non-outlier max min^{-1} values and circles outliers.

DISCUSSION

The main findings of this study were significant interpopulational variation in total sperm length of *A. arbustorum*, with a decrease from west to east, and a strong influence of parasitic mites on sperm length both within and between snail populations. A previous study has documented some level of variation in sperm length in four populations of *A. arbustorum* located within 150 km with measurements that were comparable to ours (Minoretti & Baur, 2006). The 23 sampling sites of the present study covered almost the entire distribution range of the species, with the exception of the British islands (Kerney & Cameron, 1979). The absolute differences in total sperm length among the 23 populations studied were relatively large (91.5 µm or 11.0% of the total sperm length between the shortest and longest population means), suggesting that diverse selection pressures might shape sperm length at different sites.

Several studies have demonstrated a substantial intraspecific variation in sperm length in other taxa

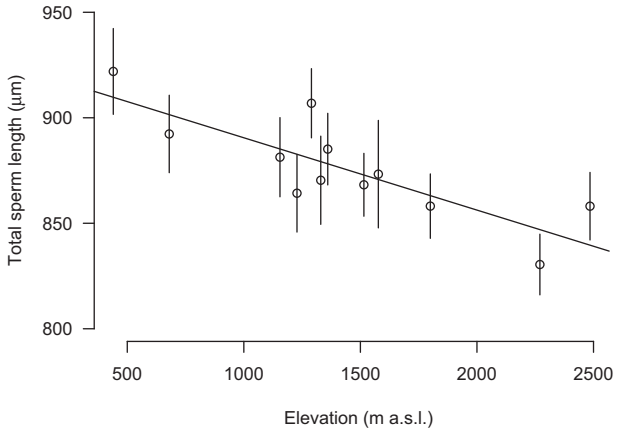

Figure 5. Relationships between total sperm length of *A. arbustorum* in mountain populations (grand mean ± SD) and elevation (R^2 = 0.675, N = 12, P = 0.001). The populations nos. 12–23 are considered (see Table 1).

(e.g., mite: Radwan, 1996; rove beetle: Green, 2003; red-winged blackbird: Lüpold *et al*., 2011), including examples in which sperm size variation appears to be relevant to male success in sperm competition (e.g., LaMunyon & Ward, 1998). In fact, the evolutionary role of post-copulatory sexual selection in shaping male reproductive traits, including sperm length and other ejaculate traits, is well documented in several taxa (Snook, 2005). However, to date most studies have focused on interspecific differences in sperm length, probably because variation between species is generally assumed to be larger than within species (Ward, 1998; Morrow & Gage, 2001).

A parasite-induced risk of mortality might increase reproductive effort, as shown in males of *Drosophila nigrospiracula* (Polak & Starmer, 1998). We found that snails infected by mites produced larger sperm than uninfected individuals, irrespective of shell size. The lack of any influence of parasitic infection on shell width in *A. arbustorum* can be explained by the fact that many individuals become infested after having attained adult size. Parasitic mites impose a physiological cost to the female function in that snails produce fewer eggs than uninfected individuals (Schüpbach & Baur, 2008b). Infected snails also show reduced activity and have higher winter mortality than parasite-free individuals (Schüpbach & Baur, 2008a, b, 2010). The mites' influence on male fitness of this hermaphrodite, through sperm production and remating interval, has not yet been studied. The larger sperm produced by infected snails suggests a parasite-induced alteration of sex allocation, by investing more into the male function. This is in accordance with theory of sex allocation in hermaphrodites (Charnov, 1996). These models consider how various aspects of sperm competition, such as mating frequency, sperm digestion and different mechanisms of sperm displacement affect sex allocation in simultaneous hermaphrodites (Charnov, 1996; Greeff & Michiels, 1999). In order to improve their situation in sperm competition, males mating in disfavoured roles are expected to put a higher investment in ejaculate and sperm quality compared with males mating in favoured roles (Parker, 1990, 1993).

The elevational decrease in sperm length could at least partly be explained by the lack of parasitic mites in alpine populations, because mites do not occur in snails living at elevations above 1500 m (Baur & Baur, 2005). There is experimental evidence that mites do not survive the long hibernation period of host snails in alpine sites (Haeussler *et al*., 2012). In *A. arbustorum*, shell size as well as egg and clutch size decrease with increasing elevation (Baur, 1984; Baur & Raboud, 1988; Baur & Baur, 1998). When differences in shell size were taken into account, correlations between elevation and egg and clutch size disappeared (Baur & Baur, 1998), but not that between sperm length and shell size (present study). Independent of parental shell size, the nitrogen concentration in eggs decreases with elevation, which may affect growth, size and survival of hatchlings (Baur & Baur, 1998). This suggests a shortage of nitrogen in snails living at high elevation, which could result in shorter sperm. However, other explanations including a reduced intensity of sperm competition at high elevation, smaller sperm storage organs (Beese, Beier & Baur, 2006) and the lack of parasitic mites at high elevation could account for the elevational gradient in total sperm length. Again, 'elevation' should be considered as a surrogate for environmental variables (temperature, amount of precipitation, etc.) that change along an elevational gradient.

In the present study, differences in total sperm length were less pronounced among individuals within populations (23.4% of the variance) than differences among populations (62.9%). Similar results were reported from eight populations of the fruit fly *Drosophila mojavensis* distributed throughout its entire geographical range of the Sonoran Desert: 26.5% of the variance in sperm length was attributable to differences among individuals within populations, and 65.0% to among-population variation (Pitnick *et al*., 2003). This suggests stabilizing selection within a population as a possible mechanism for producing variation in sperm length among populations.

It is often assumed that sperm length influences sperm swimming velocity and thus fertilization success. In *A. arbustorum*, no association was found between sperm length and sperm velocity in individuals from two populations (Minoretti & Baur, 2006). However, we cannot eliminate the possibility that snail populations differ in intensity of selection on

both pre- and post-copulatory traits and that these could be associated in one way or another.

The ability of sperm to stay and survive in the female storage organ may influence fertilization success, suggesting that optimal sperm morphology may maximize sperm longevity. The sperm storage organ (spermatheca) of terrestrial gastropods has a complex structure and it functions in the context of intense sperm competition (Baur, 1998; Chase & Darbyson, 2008; Beese *et al*., 2009). In *A. arbustorum*, sperm from different mating partners are stored in the spermatheca for months or even years before being used to fertilize eggs (Baur, 1988; Kupfernagel *et al*., 2010). In this way the processes of insemination and fertilization are uncoupled in space and time. Among the sperm stored, those with their heads in tight contact with the epithelial walls survive best (in *Cornu aspersum*; Chase & Darbyson, 2008). It has also been suggested that attachment may provide an anchor to prevent sperm from being removed from the tubule, either by passive drift or as a consequence of flagellar beating by later arriving sperm (Giusti & Selmi, 1985; Rogers & Chase, 2002). Furthermore, the beating of the flagella of sperm stored from the first mate could provide paternity assurance through increased resistance to incoming sperm from subsequent mates (Rogers & Chase, 2002), with longer sperm and larger number of sperm resulting in a stronger resistive force (Beese *et al*., 2006). Thus longer sperm might have a higher fitness.

In *A. arbustorum*, sperm delivered by the same individuals in 2–4 matings over two reproductive seasons did not differ in total length, indicating a high repeatability of this trait (Minoretti, Stoll & Baur, 2013). This suggests a genetic determination of sperm length. On the other hand, the impact of parasitic mites on total sperm length indicates a non-genetic effect. The results of a breeding experiment, in which full-siblings of *A. arbustorum* were raised at different temperatures, revealed both environmental and – to a minor extent – genetic effects on sperm length, suggesting that this trait might be affected by temperature (Minoretti *et al*., 2013). At the continental scale, we found a decrease in total sperm length from west to east. It is important to note that elevation, latitude and climatic characteristics (mean annual temperature and annual precipitation) of the population sites were highly intercorrelated and thus, only longitude, elevation, shell width and infection by mites were included in model selection.

Sperm head length varied also among populations. However, it was not influenced by any of the factors examined. As explained in the Methods, sperm head length is extremely difficult to measure because there are no sharp distinctions between head, midpiece and tail. This results in a relatively low repeatability in the measurements of sperm head length of 0.80. Thus, any interpretation of interpopulation differences in sperm head should be made with caution.

To sum up, our results suggest that selection pressures acting among populations may differ from those acting within. The reduced variation in sperm length observed among individuals with populations might be a result of stabilizing selection.

ACKNOWLEDGEMENTS

We thank A. Baur, P. Haeussler, J. Pizá and F. Reinartz for field assistance and A. Baur and three anonymous referees for valuable comments on the manuscript. Financial support was received from the Swiss National Science Foundation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Correlation matrix of characteristics of sampling sites of *A. arbustorum*. Values highlighted by bold are significant at $P = 0.05$.

SHARED DATA

Data deposited in the Dryad digital repository (Haeussler, Schmera & Baur, 2014).

Chapter III

Breeding system, shell size and age at sexual maturity affect sperm length in stylommatophoran gastropods

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BMC Evolutionary Biology, 16(1), December 2016

Schmera et al. BMC Evolutionary Biology (2016) 16:89
DOI 10.1186/s12862-016-0661-9

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Breeding system, shell size and age at sexual maturity affect sperm length in stylommatophoran gastropods

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Abstract

Background: Sperm size and quality are key factors for fertilization success. There is increasing empirical evidence demonstrating that sperm form and function are influenced by selective pressures. Theoretical models predict that sperm competition could favour the evolution of longer sperm. In hermaphrodites, self-fertilizing species are expected to have shorter sperm than cross-fertilizing species, which use sperm stored from several mating partners for the fertilization of their eggs and thus are exposed to intense sperm competition. We tested this hypothesis by comparing original data on sperm length in 57 species of simultaneously hermaphroditic stylommatophoran gastropods from Europe and South America with respect to the species' breeding system. We used *28S* rRNA nuclear and *COI* mitochondrial sequence data to construct a molecular phylogeny. Phylogenetic generalized linear models were applied to examine the potential influence of morphological and life-history characters.

Results: The best-fit model revealed that the breeding system and age at sexual maturity influence sperm length in gastropods. In general, species with predominant cross-fertilization had longer sperm than species with predominant self-fertilization or a mixed breeding system. Across species with shells (snails), sperm length also increased with shell size.

Conclusions: Our study provides evidence that sperm length in stylommatophoran gastropods is influenced by the risk of sperm competition, as well as by age at sexual maturity and shell size. This finding extends present knowledge of sperm evolution to a group of so far poorly studied simultaneous hermaphrodites.

Keywords: Comparative approach, Gastropods, Phylogeny, Sexual selection, Sperm competition, Sperm evolution

Background

There exists enormous variation in the size of sperm across the animal kingdom, ranging from 0.008 mm in the wasp *Meteorus* sp. (Hymenoptera) [1] to 58 mm in *Drosophila bifurca* [2]. Sperm size and quality are key factors for fertilization success [3–5]. Yet, the adaptive significance of variation in sperm size remains poorly understood [3]. Sperm size is expected to be selected both by sperm competition and the fertilization environment [6, 7], e.g., the female reproductive tract in the case of internally fertilizing species. 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 organs once ovulation takes place [8, 9].

Theory predicts that sperm size can increase with sperm competition risk when longer sperm achieve a higher fertilization success than shorter sperm [10, 11]. However, empirical results for internal fertilizers are conflicting. Positive relationships between sperm length and sperm competition risk have been found across nematodes [12], butterflies [13], moths [14], and frogs [15], but not in Scathophagidae (flies) [16], while the results from studies of birds vary among taxa [17]. In mammals, larger species exhibit stronger selection on sperm number than on sperm length compared to smaller species [18]. As in most taxa, our understanding of how sperm competition influences sperm size and

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structure is hampered by lack of understanding of sperm function [4, 19].

Stylommatophoran gastropods (land snails and slugs) show a huge variation in sperm length and structure, which is frequently used as a taxonomic character [20–24]. Sperm are monomorphic in all species so far examined, except in the slug *Arion ater*, which produces eupyrene and apyrene sperm [25]. The adaptive significance of the interspecific variation in gastropod sperm length, however, has not been examined.

All stylommatophoran gastropods are simultaneous hermaphrodites and numerous species reproduce predominantly by cross-fertilization [26–28]. Available evidence indicates that these gastropods copulated with different mating partners (e.g., 2–6 times per year in *Helix pomatia* [29], 2–7 times in *Cornu aspersum* [30]), resulting in multiple paternity in egg batches [31, 32], with 2–7 contributing fathers [33, 34]. Self-fertilization is also widespread, while other species have a mixed breeding system [35–37]. Within species, however, geographic and age-dependent variation in frequency of self-fertilization might occur [36, 38]. Thus, some species reproduce predominantly by self-fertilization [36–38].

Cross-fertilizing species store the sperm received in the spermatheca, which has a complex structure with several tubules and functions in the context of sperm competition [32, 39–41]. Sperm from different mating partners can be stored for months or even years before being used to fertilize eggs [42]. During copulation, sperm masses or spermatophores containing spermatozoa are reciprocally transferred into the vagina of the partner [43]. The spermatophore is transported in the reproductive tract of the recipient towards the bursa copulatrix, where it is eventually digested. During a relatively short period sperm leave the spermatophore and travel up the spermoviduct to reach the spermatheca, where they are stored until fertilization [44]. The vast majority of sperm (99.98 % in *Cornu aspersum* [45]), however, is transferred into the bursa copulatrix. Sperm stored with their heads in tight contact with the epithelial walls of the spermatheca survive best [46]. Rogers and Chase [47] suggested that the unified beating of the flagella of sperm stored from the first mate could provide paternity assurance through increased resistance to incoming sperm from subsequent mates, with longer and more numerous sperm resulting in a stronger resistive force [48]. Thus, sperm of cross-fertilizing species with multiple mating and sperm storage are exposed to intense sperm competition. In contrast, self-fertilizing individuals do not store any sperm from mating partners. Instead, they use for fertilization their own sperm passing the ova in the fertilization pouch [37, 49]. Sperm competition is absent in species reproducing exclusively by self-fertilization and strongly reduced in species reproducing predominantly by self-fertilization. We therefore hypothesize that stylommatophoran species with predominant cross-fertilization have longer sperm than species that reproduce exclusively or frequently by self-fertilization.

Sperm size, however, can also be influenced by the fertilization environment [6, 7]. There is a huge variability in the complexity of the sperm-storage organ in stylommatophoran gastropods ranging from a simple spermatheca consisting of one tubule to highly structured spermatheca with multiple tubules, while other species do not possess a sperm-storage organ [41, 43]. A comparative study showed that carrefour length (total length of spermatheca and fertilization pouch) in 17 gastropod species possessing a spermatheca was positively correlated with sperm length [41]. This suggests that sperm length in gastropods may not only be influenced by sperm competition but also by the morphology of the female reproductive tract.

In this study, we used original data on total sperm length from 57 terrestrial gastropod species occurring in Europe and South America and literature data on their breeding system to test the hypothesis that stylommatophoran species with cross-fertilization have longer sperm than species with self-fertilization. We used *28S* rRNA nuclear and *COI* mitochondrial sequence data to construct a molecular phylogeny. Some of the among-species variation in sperm length may be explained by allometry [7]. We therefore examined the effect of shell size across land snail species, taking into account phylogenetic inertia. Furthermore, large interspecific differences in morphology, anatomy, physiology, behaviour, egg morphology and the fertilization environment may confound results of among-species comparisons [50]. We used phylogenetical generalized linear models to explore how the breeding system, age at sexual maturity, lifespan, mode of reproduction (oviparous vs. ovoviviparous) and habitat preference might explain sperm length in 57 stylommatophoran species.

Results

Interspecific variation in sperm traits

The gastropod species examined differed significantly in total sperm length with mean values ranging from 101.4 μm to 1340.9 μm (Table 1; $F_{56,94} = 3093.3$, $p < 0.001$). Similarly, the species differed in the mean head length of their sperm (range: 5.6–13.8 μm; Table 1; $F_{56,94} = 48.9$, $p < 0.001$). Sperm head length was correlated with total sperm length in the species examined ($r = 0.77$, $n = 57$, $p < 0.001$). Sperm head length expressed as percentage of total sperm length varied across species from 0.9 to 7.6 % (Table 1; $F_{56,94} = 280.3$, $p < 0.001$). Considering only snails, the relative sperm length (total

Table 1 Sperm characteristics of the terrestrial gastropod species examined

Family	Species	Total sperm length (μm)	Sperm head length (μm)	Sperm head length in % of total sperm length	Relative sperm length (in % of maximum shell dimension)
Succineidae	*Succinea putris* (Linnaeus 1758)	478.8 ± 2.47	9.3 ± 0.13	1.9 ± 0.03	2.9 ± 0.02
Chondrinidae	*Chondrina avenacea* (Bruguière 1792)	374.6 ± 5.23	6.0 ± 0.40	1.6 ± 0.09	6.3 ± 0.07
	Chondrina clienta (Westerlund 1883)	336.6 ± 1.41	5.6 ± 0.34	1.7 ± 0.09	5.7 ± 0.02
	Abida secale (Draparnaud 1801)	375.1 ± 2.71	6.7 ± 0.31	1.8 ± 0.08	5.4 ± 0.04
Lauriidae	*Lauria cylindracea* (Da Costa 1778)	474.2 ± 4.01	11.7 ± 0.17	2.5 ± 0.05	13.2 ± 0.11
Orculidae	*Orcula dolium* (Draparnaud 1801)	431.9 ± 4.68	9.5 ± 0.17	2.2 ± 0.06	6.1 ± 0.06
Pyramidulidae	*Pyramidula pusilla* (Vallot 1801)	308.0 ± 7.46	8.6 ± 0.40	2.8 ± 0.07	13.2 ± 0.27
Vertiginidae	*Vertigo pygmaea* (Draparnaud 1801)	207.9 ± 1.31	8.8 ± 0.49	4.2 ± 0.24	11.3 ± 0.06
	Columella columella (Martens 1830)	176.6 ± 5.29	8.9 ± 0.43	5.0 ± 0.18	6.2 ± 0.18
Enidae	*Ena montana* (Draparnaud 1801)	590.7 ± 6.41	11.8 ± 0.23	2.0 ± 0.06	4.2 ± 0.04
Clausiliidae	*Clausilia rugosa* (Draparnaud 1801)	689.5 NA	8.2 NA	1.2 NA	9.9 NA
	Clausilia bidentata (Strøm 1765)	781.8 ± 1.15	7.5 ± 1.14	1.0 ± 0.02	7.4 ± 0.01
	Macrogastra plicatula (Draparnaud 1801)	1209.8 NA	13.0 NA	1.1 NA	9.1 NA
	Macrogastra ventricosa (Draparnaud 1801)	1195.4 NA	13.2 NA	1.1 NA	6.2 NA
	Cochlodina laminata (Montagu 1803)	1040.3 ± 1.47	10.7 ± 0.21	1.0 ± 0.02	7.1 ± 0.01
	Cochlodina fimbriata (Rossmässler 1835)	1119.5 ± 5.94	10.1 ± 0.28	0.9 ± 0.03	7.4 ± 0.04
	Balea perversa (Linnaeus 1758)	751.7 ± 0.45	8.7 ± 0.33	1.2 ± 0.05	8.5 ± 0.01
	Balea biplicata (Montagu 1803)	1061.9 ± 10.95	12.6 ± 0.19	1.2 ± 0.02	6.2 ± 0.07
Bothriembryontidae	*Discoleus aguirrei* (Doering 1884)	1215.9 ± 11.64	11.3 ± 0.50	0.9 ± 0.05	6.4 ± 0.05
	Discoleus ameghinoi (von Ihering 1908)	1232.6 ± 10.73	10.7 ± 0.50	0.9 ± 0.05	5.9 ± 0.05
Odontostomidae	*Plagiodontes patagonicus* (d'Orbigny 1835)	1340.9 ± 11.48	13.8 ± 0.15	1.0 ± 0.02	6.7 ± 0.05
	Cyclodontina (Ventania) avellanedae (Doering 1881)	908.0 ± 11.36	12.6 ± 0.37	1.4 ± 0.04	4.1 ± 0.08
Strophocheilidae	*Austroborus lutescens dorbignyi* (Doering 1876)	1050.7 ± 5.68	11.8 ± 0.30	1.1 ± 0.03	3.5 ± 0.02
Discidae	*Discus rotundatus* (Müller 1774)	429.4 ± 3.48	9.0 ± 0.17	2.1 ± 0.02	7.6 ± 0.05
Oxychilidae	*Oxychilus navarricus helveticus* (Blum 1881)	101.4 ± 0.65	7.7 ± 0.11	7.6 ± 0.16	1.3 ± 0.01
	Oxychilus draparnaudi (Beck 1837)	188.9 ± 1.61	8.1 ± 0.02	4.3 ± 0.03	1.8 ± 0.01
	Aegopinella nitens (Michaud 1831)	103.6 ± 2.29	7.6 ± 0.39	7.3 ± 0.33	1.2 ± 0.02
Zonitidae	*Zonitoides nitidus* (Müller 1774)	185.3 ± 3.96	9.3 ± 0.10	5.0 ± 0.10	3.2 ± 0.06

Table 1 Sperm characteristics of the terrestrial gastropod species examined *(Continued)*

Limacidae	*Limax maximus* Linnaeus 1758	242.4 ± 1.52	8.1 ± 0.40	3.4 ± 0.14	–
	Limax tenellus Müller 1774	160.2 ± 1.46	6.3 ± 0.07	3.9 ± 0.08	–
	Limax cinereoniger Wolf 1803	289.7 ± 0.66	8.4 ± 0.15	2.9 ± 0.06	–
Agriolimacidae	*Deroceras reticulatum* (Müller 1774)	119.5 ± 0.59	7.9 ± 0.10	6.6 ± 0.06	–
Vitrinidae	*Vitrina pellucida* (Müller 1774)	201.2 NA	5.8 NA	2.9 NA	6.7 NA
	Vitrinobrachium breve (Férussac 1821)	267.0 NA	7.6 NA	2.9 NA	5.0 NA
Arionidae	*Arion (ater) rufus* (Linnaeus 1758)	327.8 ± 3.99	7.0 ± 0.22	2.1 ± 0.09	–
	Arion vulgaris (Moquin-Tandon 1855)	340.7 NA	6.6 NA	1.9 NA	–
	Arion distinctus (Mabille 1868)	366.3 NA	6.6 NA	1.8 NA	–
Helicidae	*Helix pomatia* Linnaeus 1758	1007.8 ± 11.43	12.8 ± 0.34	1.3 ± 0.02	2.4 ± 0.03
	Cepaea nemoralis (Linnaeus 1758)	744.2 ± 2.15	10.7 ± 0.52	1.4 ± 0.07	3.1 ± 0.01
	Cepaea hortensis (Müller 1774)	767.8 ± 5.41	10.9 ± 0.21	1.4 ± 0.03	4.1 ± 0.03
	Cepaea vindobonensis (Férrusac 1821)	1180.5 ± 11.92	13.8 ± 0.47	1.2 ± 0.05	5.3 ± 0.05
	Cornu aspersum (Müller 1774)	671.6 ± 6.15	10.5 ± 0.08	1.6 ± 0.003	2.0 ± 0.02
	Eobania vermiculata (Müller 1774)	1071.1 ± 11.31	11.1 ± 0.48	1.0 ± 0.05	3.7 ± 0.04
	Theba pisana (Müller 1774)	763.2 ± 2.68	8.5 ± 0.07	1.1 ± 0.01	4.1 ± 0.01
	Arianta arbustorum (Linnaeus 1758)	847.9 ± 5.40	9.6 ± 0.16	1.1 ± 0.03	5.1 ± 0.03
	Helicigona lapicida (Linnaeus 1758)	614.2 ± 3.83	7.1 ± 0.08	1.2 ± 0.02	3.8 ± 0.02
	Isognomostoma isognomostomos (Schröter 1784)	634.0 ± 3.32	9.9 ± 0.17	1.6 ± 0.02	7.1 ± 0.03
Bradybaenidae	*Fruticicola fruticum* (Müller 1774)	337.7 ± 6.42	9.8 ± 0.06	2.9 ± 0.04	1.8 ± 0.03
Cochlicellidae	*Cochlicella acuta* (Müller 1774)	332.7 ± 4.10	5.7 ± 0.13	1.7 ± 0.06	2.4 ± 0.02
Helicodontidae	*Helicodonta obvoluta* (Müller 1774)	610.1 ± 3.60	7.2 ± 0.29	1.2 ± 0.04	4.9 ± 0.03
Hygromiidae	*Helicella itala* (Linnaeus 1758)	320.0 ± 3.58	8.0 ± 0.12	2.5 ± 0.01	2.5 ± 0.02
	Candidula intersecta (Poiret 1801)	248.3 NA	7.6 NA	3.1 NA	2.1 NA
	Xerolenta obvia (Menke 1828)	313.4 ± 3.67	8.9 ± 0.07	2.9 ± 0.06	5.5 ± 0.02
	Monachoides incarnatus (Müller 1774)	490.4 NA	7.3 NA	1.5 NA	3.6 NA
	Trochulus villosus (Studer 1789)	345.0 ± 1.60	7.9 ± 0.08	2.3 ± 0.02	2.7 ± 0.01
	Trochulus sericeus (Draparnaud 1801)	319.5 ± 0.63	7.3 ± 0.01	2.3 ± 0.01	3.5 ± 0.01
	Monacha cartusiana (Müller 1774)	347.4 ± 0.33	7.0 ± 0.31	2.0 ± 0.09	2.3 ± 0.002

Mean values ± s.e. are presented. s.e. indicate inter-individual variation. *NA* not applicable (only one individual examined). Sample sizes are given in Additional file 3

sperm length divided by the maximum shell dimension of the sperm donor) varied from 1.3 to 13.2 % among species (Table 1; $F_{49,94} = 280.3$, $p < 0.001$).

Phylogenetic tree and mode of evolution

The Maximum-Likelihood (ML) reconstruction for the combined data (both gene sections) was conducted with the best-fit model of substitution using JModelTest. The best fit model was TVM + I + G (freq. A = 0.4042; freq. C = 0.1691; freq. G = 0.1510; freq. T = 0.2757; R(a) = 0.4471; R(b) = 3.8949; R(c) = 0.7740; R(d) = 1.8532; R(e) = 3.8949; R(f) = 1; proportion of invariable sites = 0.3650; gamma distribution shape parameter = 0.3530). The ML analysis resulted in a relatively well-resolved topology (Fig. 1) and the Bayesian inference analysis (BI) produced very similar topologies.

The phylogenetic relationships of the different families were not well resolved (no bootstrap support in most cases; Fig. 1). However, most families were monophyletic. In those families, which appear not to be monophyletic, the bootstrap support was limited.

The occurrence of predominant self-fertilization and/or a mixed reproductive system was found in several distantly related families (e.g., Arionidae, Clausiliidae, Limacidae). The analysis of the ancestral state reconstruction using BayesTraits revealed that self-fertilization is the ancestral state (probability = 99.66 %). This suggests a very old and common origin (not necessarily used by all current species) and implies that at least five origins of mixed mating systems, two origins of self-fertilization and one loss of self-fertilization (a total of 8 transitions) are needed to explain the current status of the species analysed.

For the variables shell type, breeding system, mode of reproduction, age at sexual maturity, lifespan and habitat specificity, Pagel's λ ranged from 0.88 to 0.93 when all gastropod species were considered (Additional file 1), indicating some phylogenetic dependence of the traits. Considering only snails, λ ranged from 0.87 to 0.93 (Additional file 1). In all cases, λ was significantly different from both 0 and 1, suggesting an evolution model that is different from Brownian motion.

Effects of breeding system and life-history traits

Considering phylogenetic information of the gastropod species examined, the best-fit model (with the lowest AICc) revealed that sperm length was affected by both the breeding system and the age at sexual maturity (Tables 2 and 3). Delta AICc values and Akaike weights did not support any alternative model (Tables 2 and 3). The most likely model (Gast1) showed that gastropod species with predominant cross-fertilization had longer sperm than species with a mixed breeding system and species with predominant self-fertilization (Fig. 2). Phylogenetic uncertainty had only a minor effect on this pattern (Fig. 3). The most likely model also showed that species whose individuals need more than one year to attain sexual maturity had significantly longer sperm than other gastropod species whose individuals reach sexual maturity earlier (Fig. 4). Phylogenetic uncertainty had only a small effect on this pattern (Fig. 5).

Considering exclusively phylogenetic information of the snail species examined, several models showed that maximum shell dimension had a strong impact on sperm length (Tables 4 and 5). The most likely model (Snail1) revealed that sperm length of snails was affected by maximum shell dimension and age at sexual maturity (Tables 4 and 5). This model suggests that total sperm length increases with increasing shell size and that the relationship is only weakly influenced by phylogenetic uncertainty (Fig. 6). Other, still very likely models (Snail2, Snail3 and Snail 4) included also effects of the breeding system, habitat specificity and reproductive mode (Tables 4 and 5).

In gastropods, none of the proposed models received strong support for determining sperm head length (Table 6). The most likely candidate models included effects of age at sexual maturity, breeding system, lifespan, reproductive mode and shell type. In snails, the three most likely models indicate the importance of maximum shell size for determining sperm head length (Table 7).

Discussion

Our study showed that sperm length in the gastropod species examined was influenced by the breeding system and age at sexual maturity in all models. Species with cross-fertilization had longer sperm than species with self-fertilization or a mixed breeding system. In the models that considered exclusively snail species, sperm length was also affected by shell size. We used exclusively original data on sperm length measured by the same person in the same way, which excludes unexplained variation owing to different measurement techniques applied in different studies. We also present data on sperm length in tiny snails with a shell height of 1.9–3.7 mm (Table 1, Additional file 2). Surprisingly, these snails have in relation to their shell size relatively long sperm (e.g. *Vertigo pygmaea* with a shell height of 2.0 mm has on average 207.9 μm long sperm).

Female reproductive tract

In species with internal fertilization and multiple mating, sperm size is expected to be selected both by the female reproductive tract (the fertilization environment) and by sperm competition [5, 7, 41, 48]. In a study comparing 17 terrestrial gastropod species, sperm length was found to be positively correlated

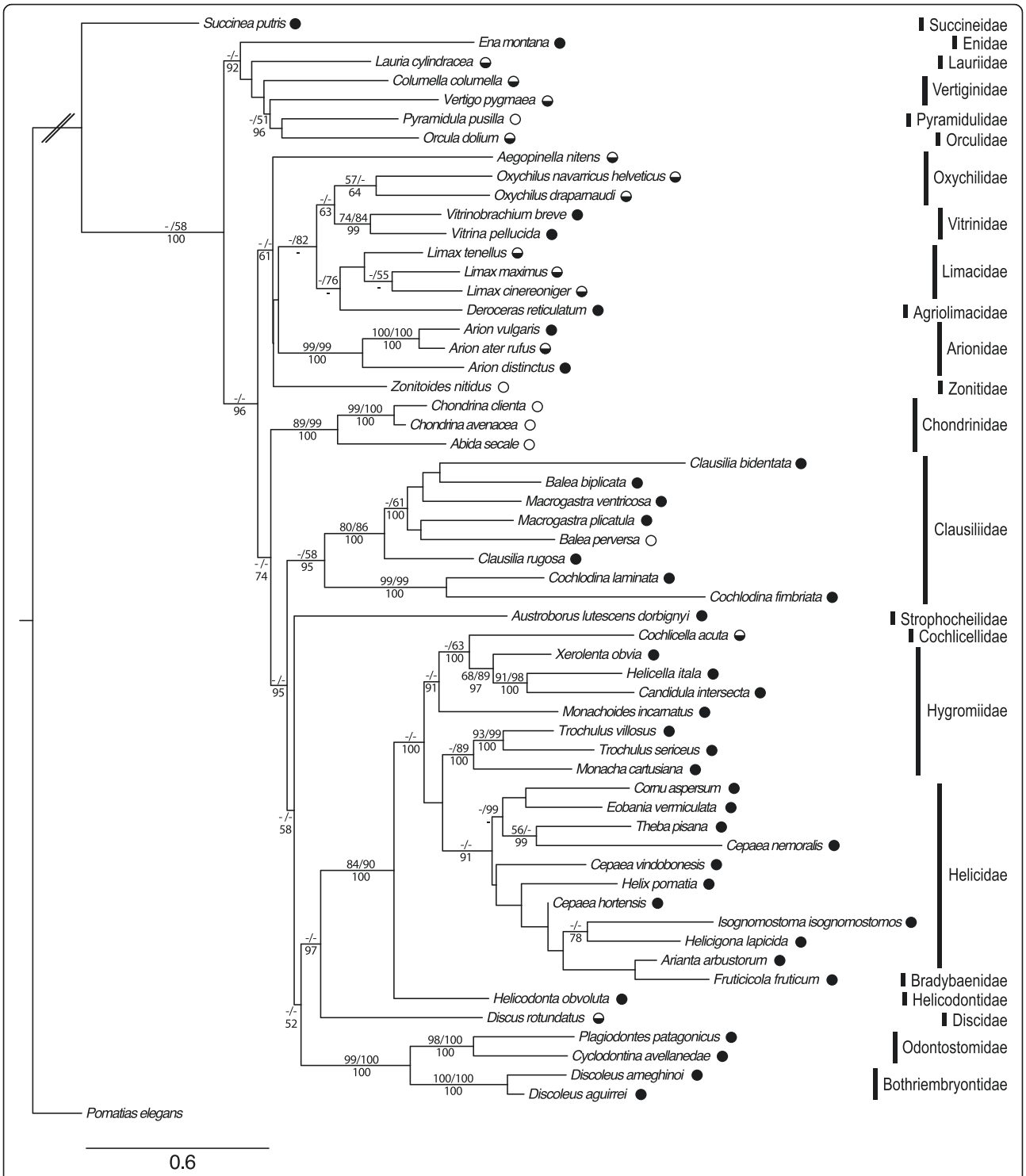

Fig. 1 Maximum likelihood tree of the concatenated sequences (*28S* and *COI*). The tree was obtained using PhyML v3.0 [97]. Sequences of 58 terrestrial gastropod species (the prosobranch *Pomatias elegans* served as outgroup) were considered. Values of bootstrap support higher than 50 % are shown for Maximum Parsimony (higher left), Maximum Likelihood (higher right) and Bayesian Inference (lower). Families and the breeding system are indicated (full circle: predominant cross-fertilization; half empty circle: mixed reproductive strategy; empty circle: predominant self-fertilization)

with the total length of the spermatheca and fertilization pouch after controlling for differences in shell size [41]. This finding supports the suggestion that the divergence in sperm length results from sperm size evolving in response to changing female reproductive tract [51]. Indeed, experimental evolution

Table 2 Best fit phylogenetic generalized linear models (ΔAICc < 3) explaining sperm length in gastropods

Model	Model specification	AICc	ΔAICc	Weight
Gast1	Sperm length ~ 1 + Age + Breeding	773.0	0.00	0.456
Gast2	Sperm length ~ 1 + Age + Breeding + Shell type	775.2	2.19	0.152
Gast3	Sperm length ~ 1 + Age	775.5	2.45	0.134
Gast4	Sperm length ~ 1 + Age + Breeding + Lifespan	775.5	2.50	0.131
Gast5	Sperm length ~ 1 + Age + Breeding + Reproduction	775.6	2.56	0.127

Age: age at sexual maturity; Breeding: breeding system; Reproduction: reproductive mode
Sample size: 57 species

Fig. 2 Effect of the breeding system on sperm length in gastropods. Phylogenetic effects and the influence of age at sexual maturity have been taken into account. Bars indicate mean values, whiskers standard errors. Different letters indicate significant differences between groups with different breeding systems (Tukey test, *p*-value adjusted following Westfall [115]). Sample sizes are 6, 13 and 38 from left to right

studies showed that changing female spermatheca length can drive the divergence in sperm length in *Drosophila melanogaster* [52, 53].

In helicid land snails, the sperm received are stored in an ordered manner in the spermatheca, usually at the bulbous blind ends of the tubules and with their heads in tight contact with the spermathecal epithelium [47, 54]. It has been suggested that the beating of the flagella of sperm from the first mate could provide paternity assurance through increased resistance to incoming sperm from subsequent mates [46, 47], with longer sperm and a larger number of sperm resulting in a stronger resistive force [48]. Thus, longer sperm competing with sperm from other mates may have a fertilization advantage by occupying and/or retaining occupancy in the storage organ better than shorter sperm. In contrast, autosperm used for self-fertilization are obtained from the spermoviduct and/or hermaphrodite duct [37, 49], and thus are not stored in a spermatheca in competition with sperm from other mates. It is important to note that most species with predominantly self-fertilization do not possess a spermatheca [41, 55].

Table 3 ANOVA tables of the best fit phylogenetic generalized linear models using Type III sums of squares explaining sperm length in gastropods

Model	Predictor	df	Sum Sq	Mean Sq	*F*	*p*
Gast1	Age	2	3556.5	1778.2	10.48	<0.001
	Breeding	2	1530.1	765.1	4.510	0.016
	Residuals	52	9623.1	185.1		
Gast2	Age	2	1567.2	783.6	5.532	0.006
	Breeding	2	1672.3	836.1	5.903	0.005
	Shell type	3	761.4	253.8	1.792	0.161
	Residuals	49	9383.1	191.5		
Gast3	Age	2	4697.3	2348.7	10.270	<0.001
	Residuals	54	12349.6	228.7		
Gast4	Age	2	2074.4	1037.2	5.995	0.004
	Breeding	2	1530.5	765.2	4.423	0.017
	Lifespan	1	0.3	0.3	0.002	0.965
	Residuals	51	11108.9	217.8		
Gast5	Age	2	3430.0	1714.9	9.969	<0.001
	Breeding	2	1140.1	570.1	3.314	0.044
	Reproduction	1	7.9	7.9	0.046	0.831
	Residuals	51	10085.3	197.8		

Age: age at sexual maturity; Breeding: breeding system; Reproduction: reproductive mode

The physical characteristics of female sperm-storage organs may impose stabilizing selection on sperm length [56]. In gastropods with spermatheca and cross-fertilization, the potential of blocking the storage organ for sperm from future mates may in turn favour divergence in spermatheca length [48]. Longer storage organs could allow the female function to take up sperm from more mating partners and thereby to benefit from a greater control over the fertilization process [53, 57, 58]. This could result in a coevolution leading to the association between sperm length and the length of the female sperm-storage organ found across snail species [41, 59], as well as across other animal taxa including moths [14], flies [16, 57, 60], beetles [61], birds [62] and mammals [63].

In this context the intraspecific variation of sperm length is of interest. In stylommatophoran gastropods, data on intraspecific variation of sperm length are

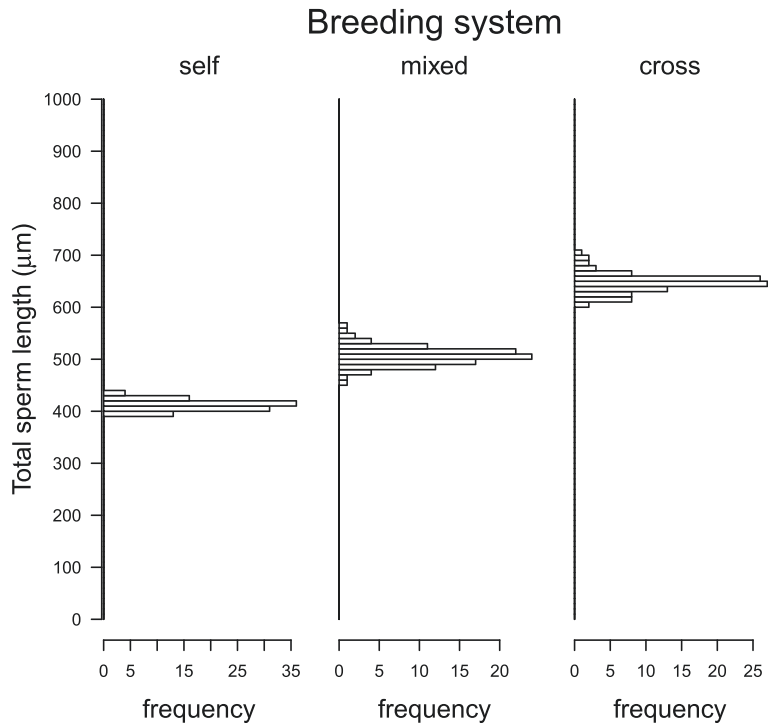

Fig. 3 Effect of phylogenetic uncertainty on how the breeding system influences total sperm length in gastropods. Histograms represent the frequency distribution of the coefficients in response to different phylogenetic trees

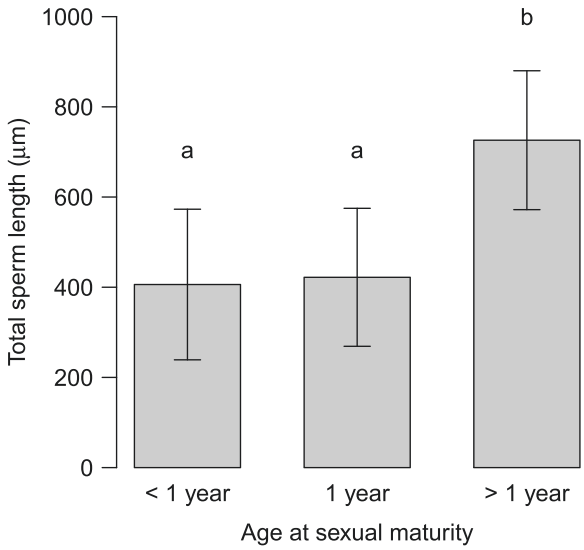

Fig. 4 Effect of age at sexual maturity on sperm length in gastropods. Phylogenetic effects and the influence of the breeding system have been taken into account. Bars indicate mean values, whiskers standard errors. Different letters indicate significant differences between age groups (Tukey test, *p*-value adjusted following Westfall [115]). Sample sizes are 5, 18 and 34 from left to right

available for only a single species [8, 64]. In 23 populations of *Arianta arbustorum* sampled across the distributional range of the species, a difference of 11 % in total sperm length between the lowest and highest population means was found [64]. Differences among *A. arbustorum* populations explained 62.9 % of the variance in total sperm length, differences among individual snails within population 23.4 % and differences within individual snail 13.7 %. Furthermore, individuals of *A. arbustorum* showed consistent sperm length in successive matings, and mean sperm length was not correlated with the number of sperm delivered in a spermatophore [8]. A breeding experiment with offspring of *A. arbustorum* raised at three different temperatures revealed both environmental and genetic effects on sperm length [65]. The relatively small intraspecific variation in this species can be contrasted with the huge interspecific variation in sperm length found in our study with sperm of *Plagiodontes patagonicus* being 13 times longer than those of *Oxychilus navarricus helveticus* (Table 1).

Sperm competition

Success in sperm competition is predicted to be influenced by variation in sperm and ejaculate quality and by interactions among competing sperm [5, 66, 67]. Sperm of cross-fertilizing terrestrial gastropod species

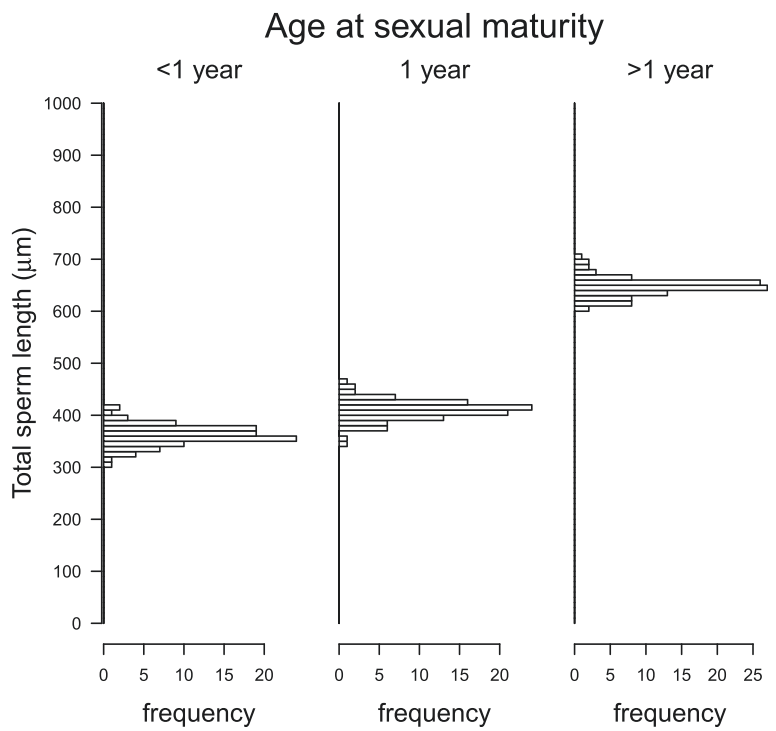

Fig. 5 Effect of phylogenetic uncertainty on how age at sexual maturity influences total sperm length in gastropods. Histograms represent the frequency distribution of the coefficients in response to different phylogenetic trees

are exposed to intense sperm competition due to multiple mating with different partners and long-term sperm storage [33, 42, 59]. Yet, apart from sperm number, relevant sperm characteristics are poorly understood in terrestrial gastropods.

Table 4 Best fit phylogenetic generalized linear models (ΔAICc < 3) explaining sperm length in snails

Model	Model specification	AICc	ΔAICc	Weight
Snail1	Sperm length ~ 1 + Age + Max	674.4	0.00	0.266
Snail2	Sperm length ~ 1 + Age + Breeding + Max	674.9	0.42	0.216
Snail3	Sperm length ~ 1 + Age + Habitat + Max	676.0	1.52	0.124
Snail4	Sperm length ~ 1 + Age + Max + Reproduction	676.4	1.92	0.102
Snail5	Sperm length ~ 1 + Breeding + Habitat + Max + Shape	676.8	2.36	0.082
Snail6	Sperm length ~ 1 + Age + Lifespan + Max	676.9	2.43	0.079
Snail7	Sperm length ~ 1 + Age + Habitat + Max + Shape	677.2	2.81	0.065
Snail8	Sperm length ~ 1 + Age + Breeding + Max + Shape	677.3	2.82	0.065

Age: age at sexual maturity; Breeding: breeding system; Habitat: habitat specificity; Max: maximum shell dimension; Shape: shell shape; Reproduction: Reproductive mode
Sample size: 50 species

In general, sperm size may influence the outcome of sperm competition through multiple mechanisms (reviewed in Simmons [19] and Pizzari and Parker [5]). In stylommatophoran gastropods, sperm size may influence: (i) the number of sperm delivered during a copulation because of a potential trade-off between sperm length and number; (ii) sperm longevity and/or swimming speed; and (iii) the defence ability of sperm stored in the spermatheca of the recipient against sperm from future mates (see above). Available evidence suggests that there is no trade-off between sperm length and the number of sperm delivered in a spermatophore. In none of the four *Arianta arbustorum* populations examined was a correlation between mean sperm length and the number of sperm delivered found after having removed the effect of shell size [8]. Sperm competition may select for longer sperm if sperm size is positively linked to sperm competitiveness through increased velocity, motility, longevity, and/or a better ability to defend their position in spermatheca [10, 66]. In *Drosophila melanogaster*, relatively long and slow sperm are at an advantage in entering or remaining in the "fertilization set" of the female during the sperm storage and displacement phase of sperm competition, thereby gaining a chance of being used for fertilization later [67].

Table 5 ANOVA tables of the best fit phylogenetic generalized linear models using Type III sums of squares explaining sperm length in snails

Model	Predictor	df	Sum Sq	Mean Sq	*F*	*p*
Snail1	Age	2	2147.4	1073.7	7.292	0.002
	Max	1	2357.7	2357.7	16.012	<0.001
	Residuals	46	6773.5	147.3		
Snail2	Age	2	1665.6	832.8	6.372	0.004
	Breeding	2	572.2	286.1	2.188	0.124
	Max	1	1479.6	1479.6	11.320	0.002
	Residuals	44	9910.3	225.2		
Snail3	Age	2	2286.7	1143.3	8.242	<0.001
	Habitat	2	450.8	225.4	1.625	0.209
	Max	1	2252.4	2252.4	16.238	<0.001
	Residuals	44	8680.5	197.3		
Snail4	Age	2	2194.2	1097.1	7.525	0.001
	Max	1	1875.2	1875.2	12.862	<0.001
	Reproduction	1	74.1	74.1	0.509	0.479
	Residuals	44	9641.5	219.1		
Snail5	Breeding	2	878.6	439.3	3.874	0.029
	Habitat	2	705.1	352.6	3.087	0.056
	Max	1	1836.6	1836.6	16.085	<0.001
	Shape	2	1840.0	920.0	8.058	0.001
	Residuals	42	7224.4	172.0		
Snail6	Age	2	1390.2	695.1	4.636	0.015
	Lifespan	1	6.4	6.4	0.042	0.837
	Max	1	2340.7	2340.7	15.613	<0.001
	Residuals	45	10214.3	226.9		
Snail7	Age	2	878.5	439.3	3.573	0.037
	Habitat	2	677.6	338.8	2.756	0.075
	Max	1	1942.7	1942.7	15.803	<0.001
	Shape	2	474.9	237.4	1.932	0.157
	Residuals	42	9000.9	214.3		
Snail8	Age	2	666.8	333.4	2.829	0.070
	Breeding	2	661.6	330.8	2.807	0.072
	Max	1	1128.8	1128.2	9.578	0.003
	Shape	2	336.6	168.3	1.428	0.251
	Residuals	42	9854.4	234.6		

Age: age at sexual maturity; Breeding: breeding system; Habitat: habitat specificity; Max: maximum shell dimension; Shape: shell shape; Reproduction: Reproductive mode

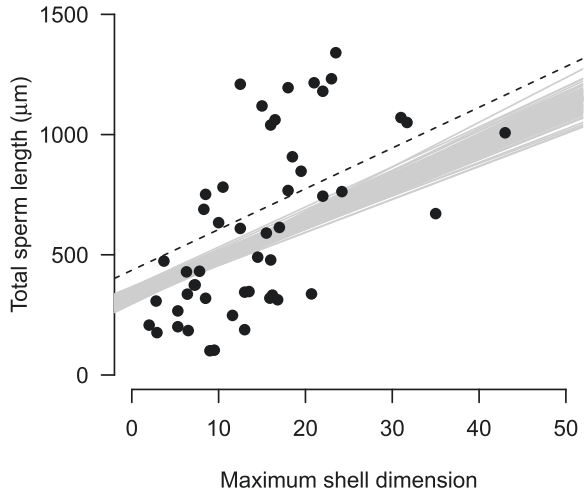

Fig. 6 Relationship between total sperm length and maximum shell dimension in snails ($n = 50$ species). The dotted line represents the cross-species regression corrected for the most likely phylogeny, while grey lines represent alternatives phylogenetic models

Sperm length in the snail *A. arbustorum* was neither correlated with mean velocity nor with percentage motility or longevity, all measured *in vitro* [8]. However, mean sperm velocity differed among individual snails (range 52–112 μm/s). Furthermore, the percentage motility and longevity of sperm differed between snails from the two populations, but were not affected by shell size [8]. After spermatophore transfer, longer sperm might have a higher probability of leaving the spermatophore and escaping the peristaltic waves of the duct transporting them to the sperm-digesting organ [44]. In *Helix pomatia* and *Cornu aspersum*, only about 0.025 % of the transferred sperm are stored in the spermathecal tubules of the storage organ [44, 45]. However, experiments are needed to test whether longer sperm have an advantage in this process.

With the unified beating of their flagella sperm stored in the spermatheca of the recipient may generate resistance to incoming sperm from subsequent mates entering the tubules and thus provide paternity assurance [47], with longer and more numerous sperm resulting in a stronger resistive force [46, 48]. This hypothesis might also explain why sperm of stylommatophoran species with a complex sperm-storage organ are longer than those of other gastropods with simple or no sperm storage organ, most of them being frequently or predominantly self-fertilizing species. Several comparative studies considering a diverse array of taxa examined the evolutionary relationship between sperm length and some estimates of risk or intensity of sperm competition (reviewed in Pitnick et al. [7]). For example, in rhabditid nematodes, males of gonochoristic species had significantly larger sperm than males of the hermaphrodite species [12]. In nematodes, males of gonochoristic species are normally exposed to a high risk of sperm competition, whereas hermaphroditic species mainly reproduce by self-fertilization resulting

Table 6 Best fit phylogenetic generalized linear models (ΔAICc < 3) explaining sperm head length in gastropods

Model	Model specification	AICc	ΔAICc	Weight
SpermHGast1	Head length ~ 1 + Lifespan	235.2	0.00	0.143
SpermHGast2	Head length ~ 1 + Age	235.6	0.43	0.116
SpermHGast3	Head length ~ 1 + Breeding + Shell type	235.6	0.46	0.114
SpermHGast4	Head length ~ 1 + Age + Breeding + Shell type	236.3	1.07	0.084
SpermHGast5	Head length ~ 1 + Age + Breeding	236.4	1.21	0.078
SpermHGast6	Head length ~ 1 + Breeding + Lifespan + Shell type	236.4	1.23	0.078
SpermHGast7	Head length ~ 1 + Age + Reproduction	236.7	1.49	0.069
SpermHGast8	Head length ~ 1 + Lifespan + Reproduction	236.7	1.55	0.066
SpermHGast9	Head length ~ 1	236.9	1.68	0.062
SpermHGast10	Head length ~ 1 + Breeding + Lifespan	236.9	1.75	0.060
SpermHGast11	Head length ~ 1 + Age + Reproduction + Shell type	237.2	2.03	0.052
SpermHGast12	Head length ~ 1 + Age + Lifespan	237.5	2.27	0.046
SpermHGast13	Sperm length ~ 1 + Lifespan + Reproduction + Shell type	238.0	2.86	0.034

Age: age at sexual maturity; Breeding: breeding system; Reproduction: reproductive mode
Sample size: 57 species

in a low sperm competition risk. The main conclusion to be drawn from the various correlational studies is that sperm competition is important in the evolutionary diversification of sperm size in some but not in all animal groups.

Allometric effects

Some of the among-species variation in sperm size may be explained by allometry: the way characters scale with shell or body size. We found a positive relationship between sperm length and shell size after having taken phylogenetic relationships into account. This finding supports the results of an earlier study on land snails with a much smaller sample size (17 species [41]) and extends our knowledge of the positive sperm length–body size relationships in other invertebrates (butterflies [13]; fruit flies [2]; nematodes [12]; an exception being dung flies [16]). Two non-mutually exclusive hypotheses have been suggested for the sperm length–body size relationships observed across some invertebrate groups [7]. First, increases in body size and sperm size may be independently favoured by sexual selection. Second, because of the energetic demands of producing relatively long sperm, sperm size and body size may coevolve. Interspecific studies with *Drosophila* have demonstrated substantial energetic costs and life history trade-offs associated with the production of relatively long sperm [2, 7, 68], while intraspecific analyses with *D. hydei* showed how larger body size mitigates those costs for males [69]. Similar studies are not available for terrestrial gastropods.

Table 7 Best fit phylogenetic generalized linear models (ΔAICc < 3) explaining sperm head length in snails

Model	Model specification	AICc	ΔAICc	Weight
SpermHSnail1	Head length ~ 1 + Max	200.0	0.00	0.432
SpermHSnail2	Head length ~ 1 + Lifespan + Max	201.3	1.34	0.221
SpermHSnail3	Head length ~ 1 + Max + Reproduction	202.3	2.25	0.140
SpermHSnail4	Head length ~ 1 + Age + Breeding + Shape	202.8	2.78	0.108
SpermHSnail5	Head length ~ 1 + Age + Breeding	202.9	2.92	0.100

Age: age at sexual maturity; Breeding: breeding system; Max: maximum shell dimension; Shape: shell shape; Reproduction: Reproductive mode
Sample size: 50 species

Advantage of self-fertilization

Simultaneous hermaphroditism is advantageous when mates are hard to find. In such situations each sexually mature conspecific encountered is a potential mating partner. Simultaneous hermaphroditism also offers opportunities for self-fertilization. Evolutionary theory predicts the conditions under which simultaneous hermaphrodites should reproduce by self-fertilization [70]. Depending on the relative balance of the costs and benefits, populations are assumed to evolve towards complete cross-fertilization or complete selfing [71]. Nevertheless, mixed mating strategies are also frequently observed in a variety of taxa in nature [72, 73].

Self-fertilization provides advantages including assurance of reproduction in the absence of mating partners, preservation of highly fit genotypes, and reduced energy allocation to both sperm production and mating behaviour [70, 71]. On the other hand, self-fertilization results in low heterozygosity, which in turn reduces the chances to adapt to changing

environmental conditions and thus enhances the risk of local extinction [74].

In stylommatophoran gastropods, the frequency of self-fertilization varies greatly among species and – to a smaller extent – even among populations within species [37, 38]. In some species, it is rare, in others it occurs occasionally, and still in others self-fertilization occurs regularly [75]. Low frequencies of self-fertilization also occur in species so far considered as obligate cross-fertilizers. For example, in a natural population of *A. arbustorum*, a low frequency of self-fertilization was found in two out of 41 mother-progeny arrays: Two mother snails produced 2.0 and 18.2 % of their offspring by self-fertilization, while the remaining 39 mother snails reproduced exclusively by cross-fertilization [76]. However, laboratory breedings showed strong effects of inbreeding depression in this species; selfing individuals had a reproductive success of 1–2 % compared with that of cross-fertilizing individuals [77]. Strong inbreeding depression was also observed in *Triodopsis albolabris* [78], another snail species that predominantly reproduces by cross-fertilization. In contrast, inbreeding depression appears to be absent in the slug *Deroceras agreste*, a species with frequent self-fertilization [37]. Self-fertilizing individuals were two to four times more fecund, had a longer lifespan and the growth rate of their offspring was higher than that of cross-fertilizing individuals. Similarly, inbreeding depression was very low in self-fertilizing *Balea perversa* [79, 80].

Within-species variation in breeding system could influence the results of our analyses. To minimize errors due to misclassified species, we used three categories of breeding systems: predominantly cross-fertilizing species, predominantly self-fertilizing species and species with a mixed breeding system. To the latter category we assigned all species in which the available information was ambiguous, i.e., some authors reported cross-fertilization but records on selfing were also found in the same species. Our analyses showed that the main differences in sperm length were between cross-fertilizing species with longer sperm and both self-fertilizing species and species with a mixed breeding system, which produced shorter sperm. Available information indicates that species with a mixed breeding system fertilize 70–80 % of their eggs by self-fertilization, suggesting less intense sperm competition than in cross-fertilizing species. This could be an explanation for the small difference in sperm length between species with predominant self-fertilization and species with a mixed breeding system.

Conclusions

In conclusion, we found evidence that sperm length in stylommatophoran gastropods is influenced by the risk of sperm competition experienced in different breeding systems, as well as by age at sexual maturity and shell size. However, female morphology (the size and structure of the sperm-storage organ) may also influence divergence in sperm length. Our findings extend present knowledge of sperm evolution in a rarely studied group of simultaneous hermaphrodites and highlight the complexity of postcopulatory processes in this group.

Methods

Gastropod species

We analysed the sperm of 57 terrestrial gastropod species (50 land snail and 7 slug species) representing 23 families of the subclass Stylommatophora (Table 1). Adult specimens were collected at various localities in Europe and South America during the reproductive seasons in 2011 and 2012 (Additional file 3). Sampling of gastropods complied with national and international guidelines. The snails and slugs collected were neither protected by law nor endangered in the countries they were collected. The gastropods sampled were immediately frozen at −80 °C. Nomenclature of gastropod families and species follows Bouchet et al. [81] and Breure and Romero [82].

Sperm length measurements

In stylommatophoran gastropods, the autosperm produced are stored in the midsection of the hermaphroditic duct, the vesicula seminalis [83]. We dissected out the hermaphroditic duct of three specimens per species (in a few species the sample size was smaller; Additional file 3). Using a fine needle, we opened the wall of the sperm-containing part of the hermaphrodite duct along its longitudinal axis and placed it for 12–24 h in 240 μl PBS with Ca^{2+}/Mg^{2+} at 4 °C. We measured sperm length following Minoretti and Baur [8]. Aliquots of 20 μl sperm suspension were placed on two microscopic slides, covered with a coverslip and sealed with translucent nail polish. We digitized randomly chosen spermatozoa using a camera (Canon PowerShot S70) mounted on a compound microscope (Leica DMLD, PH3, magnification 40–100x) connected to a Macintosh computer. From these images, we measured total sperm length (head and tail) and sperm head length for 25 spermatozoa from each specimen using ImageJ (version 1.43f; https://imagej.nih.gov/ij/). Freezing at −80 °C does not appear to affect sperm length. Sperm obtained from freshly killed individuals of *Arianta arbustorum* (never frozen) and sperm from the same individuals kept at −80 °C for 2 months did not differ in length (N. Minoretti, unpublished data).

We assessed the reliability of multiple length measurements on the same sperm (eight sperm from one individual of eight different species on 12 days) by calculating the repeatability following Lessells and Boag [84]. Repeatability of

multiple sperm length measurements was 0.97 indicating that the technique was accurate.

To adjust sperm characters to snail size, we measured shell width and shell height of each snail individual to the nearest 0.1 mm using a vernier calliper (for slug species see below).

Gastropod characteristics

Data on the predominant breeding system (cross-fertilization, self-fertilization, or a mixed system) and reproductive mode (oviparity in species that deposit eggs or ovoviviparity in species that retain fertilized eggs in the female reproductive duct) were compiled from different sources [26–28, 36, 49]. Data on life-history traits (maximum shell dimension of adult snails, age at sexual maturity and lifespan) and habitat specificity (open-land: species exclusively occurring in open habitat; forest: species mainly found in wooded areas; ubiquitous: species found in different types of habitat) were obtained from various sources [37, 38, 85–88], B. Baur (unpubl. data) and J. Pizá (unpubl. data)] (see Additional file 2). Maximum shell dimension (shell height or shell width whatever is larger in a species) has been shown to be a reasonably good surrogate for body size in terrestrial snail species with different shell shape [86, 89].

The shape of gastropod shells (oblong, globose or depressed) is of ecological significance because of the strong associations between shell shape and the angle and nature of substrate on which the snails are active [90–92]. Species with oblong shells use vertical surfaces or burrow in soft substrate, species with depressed shells predominantly occur on horizontal surfaces, while globular species are less specific in their preferences. Shell shape of snails is frequently expressed as the maximum shell height divided by the maximum shell width (hereafter shell shape index, SI [93]). Using literature data on shell height and shell width we calculated SI for each snail species and assigned it to three classes: snails with flat or depressed shells ($SI \leq 0.65$); snails with globose shells ($0.65 < SI < 1.35$); and snails with oblong shells ($SI \geq 1.35$). The classes correspond to peaks in the frequency distribution of the shell shape index reported in various terrestrial gastropod faunas [93]. In data analyses including all gastropods, we used the variable shell type, which includes the three shell shape classes of snails and as a fourth group all slug species examined (gastropods without shell).

Phylogenetic analyses

We analysed sections of the *28S* and *COI* genes to determine the phylogenetic relationships between the gastropod species examined. Sequences of the *28S* were available on GenBank or iBOL for 34 species and those of *COI* for 36 species. To complete the dataset for both genes, we extracted total genomic DNA from the foot of a specimen from each of 32 species using QIAamp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland). The *28S* gene was amplified by PCR with the primers *28S*-forward and *28S*-reverse [41] in a 25 μl volume using Taq PCR Core kit (Qiagen) with 0.5 μM of each primer, 1.5 mM of $MgCl_2$, 1x Q-solution and 1x buffer. Amplifications were conducted for 35–45 cycles (depending on the DNA quality) of 95 °C during 30 s, 50 °C during 30 s and 72 °C during 60 s. The *COI* was amplified with the primers FCOI and RCOI under the same conditions as presented above, except for $MgCl_2$ (2.5 mM) [94]. The PCR was composed of 35–45 cycles of 95 °C during 45 s, 52 °C during 45 s and 72 °C during 60 s. PCR products were checked on 1.5 % agarose gel and thereafter sent to Macrogen (Seoul, Rep. of Korea) for sequencing. All sequences were deposited in GenBank (for accession nos. see Additional file 4). We used sequences of the prosobranch land snail *Pomatias elegans* from GenBank as an outgroup.

The sequences were checked using CodonCode Aligner v 4.0.3 (CodonCode Corporation, Centerville, USA) and aligned with ClustalX v2.0 [95]. We selected the appropriate model of sequence evolution using JModelTest v2.1.5 [96] based on AIC (Akaike Information Criterion) and applied to the different phylogenetic reconstruction methods. We ran Maximum Likelihood analyses (ML) using PhyML v3.0 [97]. We applied PAUP* v4.0b.10 [98] to evaluate the Maximum Parsimony (MP) trees (heuristic searches with random stepwise addition and TBR branch swapping options). The robustness of the trees was assessed by bootstrap resampling using 1000 random MP and ML repetitions. Bayesian inference analyses (BI) were performed with MrBayes v3.12 using the $GTR + I + G$ model of substitution [99]. The analysis was run with four chains of 10^7 generations, with a sampling every 100 generations. The first 10 % of the trees were discarded as burn-in. In order to check the stability of the simulations, the different parameters were plotted using Tracer v1.6 [100]. To evaluate the effect of phylogenetic uncertainty in the morphological analyses, 100 trees of the MrBayes analysis were sampled (one tree was selected every 91,000 generations). The ancestral state of the breeding system (self-fertilization or cross-fertilization) was examined using Multistate ML with BayesTraits v2 [101] and 100 random trees obtained from the MrBayes analysis.

Data analyses

Relative sperm length was calculated based on the shell size of the sperm-producing individual (expressed in % of maximum shell dimension; slugs were excluded from this analysis). Relative sperm length indicates the length

of sperm in relation to the size of the animal and was only used for illustrative purposes (Table 1).

For among-species comparisons of total sperm length and sperm head length, we used species mean values of life-history traits obtained from the literature (see above). The maximum shell dimension of a snail was used as a measure of body size, which allowed us to test possible effects of allometry on sperm size. Extended body length of slugs cannot be compared with any measures of shell size in snails. We therefore ran all analyses twice. First, we used data of all gastropod species ($n = 57$), but did not consider body size in the analyses. Second, we used data of all snail species ($n = 50$, excluding slug species) and considered maximum shell dimension as a measure of body size in the analyses.

Snail shell width, shell height and the derived maximum shell dimension were measured on ratio scales. For all gastropods, age at sexual maturity and lifespan were expressed on ordinal scales, while breeding system, habitat specificity and reproductive mode were expressed on nominal scales. Due to problems associated with the analyses of ordinal scale data, these data were reduced to nominal data [102]. Variance inflation factors (VIF) were used to test for collinearity of traits. Gastropod traits showed only moderate collinearity (the highest observed value was observed both for gastropods and snails reaching sexual maturity at an age of 1 year with values of 3.092 and 3.121, respectively). We therefore used all traits in the statistical modelling.

Pagel's maximum likelihood (hereafter λ) was used to estimate the phylogenetic signal in our dataset. λ varies between 0 (phylogenetic independence of the data) and 1 (strong phylogenetic signal with a Brownian Motion evolution model). In cases of $0 < \lambda < 1$, a phylogenetic dependence can be assumed. Likelihood ratio tests were used to compare estimated λ with values of 0 and 1 [103, 104].

We used phylogenetic generalized linear models (PGLMs) to examine how species traits explain sperm characteristics of gastropods, taking into account phylogenetic dependence of data. We considered data on age at sexual maturity, breeding system, habitat specificity, shell type and reproductive mode for gastropod species. In models considering only snails, three groups of species with different shell shape were used (depressed, globose and oblong shells), while in models considering all gastropods slugs (gastropods without shell) were considered as a fourth group in the trait shell type. We added maximum shell dimension to these traits and replaced shell type by shell shape when snail species were examined. Only main effects without interactions were examined. We assessed the performance of PGLMs based on all possible combinations of species traits. This resulted in 64–128 different models depending on whether sperm characteristics of gastropods or snails were considered. The best-fit models were selected using an information theoretic approach (following Garamszegi & Mundry [105]) based on the Akaike Information Criterion corrected for the number of cases and parameters estimated (AICc) and Akaike weights. Delta AICc indicates the difference in the fit between a particular model considered and that of the best fit model. Models with a delta AICc < 3 are shown in the Results section. AIC weight was calculated among this subset of models. Phylogenetic uncertainty was assessed by running the best statistical model with each of the 100 phylogenetic trees (see above: Phylogenetic analyses). This resulted in a frequency distribution of the particular sperm character for each predictor (Figs. 3 and 5).

Statistical analyses were run in R [106] using the packages *ape* [107], *caper* [108], *faraway* [109], *geiger* [110], *multcomp* [111], *MuMIn* [112], *nlme* [113] and *picante* [114].

Ethics
Not applicable

Consent to publish
Not applicable

Additional files

Additional file 1: Pagel's λ and tests of data independence ($\lambda = 0$) and of Brownian Motion ($\lambda = 1$). (a) for gastropods ($n = 57$ species, slugs included), and (b) for snails ($n = 50$ species; slugs excluded). (PDF 26 kb)

Additional file 2: Shell characteristics, life-history traits and habitat specificity of the terrestrial gastropod species examined. (PDF 157 kb)

Additional file 3: Gastropod species, locations of sampling, elevation, and sampling date together with sample size. (PDF 141 kb)

Additional file 4: GeneBank accession numbers for the new nucleotide sequences. (PDF 9 kb)

Abbreviations
AIC: Akaike Information Criterion; BI: Bayesian inference analysis; ML: maximum-likelihood reconstruction; PGLM: phylogenetic generalized linear model; SI: shell shape index.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
All authors contributed to designing the study. DS performed analyses, JP collected gastropods and dissected them, ER collected gastropods, dissected them and measured sperm length, SU performed the molecular analyses, BB conceived and coordinated the study, collected gastropods and drafted the manuscript, incorporating revisions from all co-authors. All authors have read and approved the final version of the manuscript.

Acknowledgements
We thank J. Koene, E. Riedener and A. Staikou for help in snail sampling and A. Baur, B. Braschler, S. Lüpold, H.-P. Rusterholz and three anonymous reviewers for comments on the manuscript.

Funding
Financial support was received from the Swiss National Science Foundation (grant no. 31–140915 to BB).

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Received: 16 February 2016 Accepted: 18 April 2016
Published online: 29 April 2016

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Chapter IV

Random mating with respect to mating status in the simultaneously hermaphroditic land snail *Arianta arbustorum*

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Invertebrate Reproduction & Development, 2014, **58**(2): 115–123

Invertebrate Reproduction & Development
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tinv20

Random mating with respect to mating status in the simultaneously hermaphroditic land snail Arianta arbustorum

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Published online: 05 Nov 2013.

Invertebrate Reproduction & Development, 2013
http://dx.doi.org/10.1080/07924259.2013.855267

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(*Received 16 December 2012; accepted 7 October 2013*)

In promiscuous species with sperm storage, males are expected to show a preference for mating with virgin and young females to reduce the risk of sperm competition. In the simultaneous hermaphrodite land snail *Arianta arbustorum*, sperm production precedes egg production by 2–4 weeks, resulting in a short period of protandric hermaphroditism before shell growth is completed. In natural populations, copulating pairs involving individuals which have not yet completed shell growth (virgins) have been observed. We ran a series of mate-choice experiments to examine whether virgin and nonvirgin (experienced) individuals of *A. arbustorum* discriminate between virgin and nonvirgin mating partners. We also assessed the number of sperm delivered to partners with different mating status. Neither virgin nor nonvirgin snails showed any preference for mating with a virgin partner. In all test situations mating was random and the number of sperm delivered was not adjusted to the mating status of the partner. Mating success was mainly determined by the activity of the individual. The random mating pattern does not imply random fertilization of eggs because the presence of a sperm-digesting organ and the morphology of the sperm storage organ allow a selective storage and use of sperm in *A. arbustorum*.

Keywords: mate choice; mate recognition; mating behavior; preference; sexual selection

Introduction

Models of sexual selection predict that partner choice increases both average male and average female fitness in a population (Andersson 1994; Birkhead and Møller 1998). According to male mate-choice theory, males should prefer to mate with nonexperienced (virgin) females because virgins do not yet store sperm from other males, which may compete for fertilization success (Parker 1998). Thus, males mating with virgin females might have a higher reproductive success than males mating with females already storing sperm from previous mate(s). However, this prediction is based on the assumption that males are able to recognize the mating status of the potential partner and there is sperm mixing in the storage organ of multiple-mated females. In a variety of animal species, chemical and (or) age-depending cues have been reported as indicators for the mating status of potential partners (e.g. Uhía and Rivera 2005; Friberg 2006; Thomas 2011). In various insect species, males show a preference for mating with virgin or young females to reduce sperm competition risk for their own sperm and thus to increase siring success (e.g. Simmons et al. 1994; Bateman and Ferguson 2004). There is also evidence that males might provide smaller ejaculates or terminate copulation earlier when mating with females with a higher risk of sperm competition (e.g. Bukowski and Christenson 1997). In other species, however, males do not respond to the mating status of females (e.g. Elgar et al. 2003). Theory also predicts that mate-choice criteria vary with the reproductive state. Consequently, virgin females are expected to be less choosy than mated individuals (Halliday 1983).

Mechanisms of mate-choice are of particular interest in simultaneous hermaphrodites, which are functionally females and males at the same time. Thus, pre-copulatory sexual selection processes are influenced both by the female and the male traits of an individual. During simultaneous intromission and sperm exchange, a sperm donor is at the same time a sperm receiver. Consequently, a sexual conflict between female and male interests may arise within an individual (Michiels 1998).

Promiscuity and multiple paternity are well documented in simultaneously hermaphroditic land snails (Baur 1994; Rogers and Chase 2002; Evanno et al. 2005; Garefalaki et al. 2010). We designed mate-choice tests to examine whether nonexperienced (virgin) and experienced (nonvirgin) individuals of the land snail *Arianta arbustorum* discriminate between virgin and nonvirgin mating partners. We also assessed the number of sperm delivered during copulation to test whether individuals of *A. arbustorum* are able to adjust sperm expenditure to the mating status of their partner. Sperm

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competition theory predicts that the males should allocate sperm according to the number of competing ejaculates to increase confidence of paternity (Parker et al. 1997). Engqvist and Reinhold (2006) extended existing models to species with internal fertilization. Their predictions depend on the female remating rates and patterns of sperm utilization. According to these models, individuals of *A. arbustorum* mating with a virgin partner should deliver more sperm than individuals copulating with already mated partners, because there is first mate precedence in this species (Baur 1994).

A. arbustorum has determinate growth. Sexual maturity is attained at an age of 2–4 years when the shell growth is completed (Baur and Raboud 1988). However, sperm production precedes egg production by 2–4 weeks, resulting in a short period of protandric hermaphroditism in this otherwise simultaneous hermaphrodite (Luchtel et al. 1997). Matings involving individuals that had not yet finished shell growth have been observed in natural populations (Baur 1984a; Kupfernagel and Baur 2011a). *A. arbustorum* mates 1–4 times in the course of a reproductive season and fertile sperm can be stored for more than a year in the sperm storage organ (Baur 1988a), resulting in high levels of multiple paternity within clutches (Kupfernagel et al. 2010). Mate-choice experiments revealed an absence of pre-copulatory discrimination between individuals of different size and relatedness (Baur 1992; Baur and Baur 1997). It is, however, unknown whether copulating snails discriminate between partners of different mating status.

In the present study, we addressed the following questions: (1) Do virgin and nonvirgin individuals of *A. arbustorum* show a preference for mating with a virgin? (2) Do virgin and nonvirgin individuals differ in their pre-copulatory behavior and in courtship duration? (3) Do virgin and nonvirgin snails differ in the number of sperm transferred in the spermatophore and/or do they adjust the number of sperm delivered to the mating status of the partner?

Materials and methods

Study organism

A. arbustorum is common in moist habitats of northwestern and central Europe (Kerney and Cameron 1979). Adult individuals reach a shell breadth of 16–22 mm (Baur 1984b). During winter the snails hibernate in leaf litter or burried in the soil (Baur 1986). Snails reproduce for a period of 3–5 years (maximum 14 years; Baur and Raboud 1988). Mating in *A. arbustorum* includes elaborate courtship behavior, with optional dart shooting (i.e. the pushing of a calcareous dart into the mating partner's body), and lasts for 2–18 h (Hofmann 1923; Baur 1992). Copulation is reciprocal. After intromission, each snail simultaneously transfers one spermatophore (Haase and 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 of 2–3 cm (Baur et al. 1998). The spermatophore is dissolved in the female reproductive tract of the recipient within few days (Haase and Baur 1995). Only sperm that leave the spermatophore through its tail can bypass the digesting bursa copulatrix and reach the spermatheca, where they are stored (Baur 2007). In the field, snails deposit one to three egg batches per reproductive season, each consisting of 20–50 eggs (Baur and Raboud 1988; Baur 1990). Outcrossing is the dominant mode of reproduction, but self-fertilization may occur in low frequencies, particularly after long periods of isolation (Chen 1994; Kupfernagel et al. 2010).

Sampling and maintenance of test snails

Virgin snails (V) were obtained by collecting subadult individuals of *A. arbustorum* (snails that had not yet completed shell growth) in a subalpine forest near Gurnigelbad (20 km south of Bern, 46°45′ N, 7°28′ E, elevation 1230 m a.s.l.) immediately after snow melt on 2 May 2009. At the same time, fully-grown adult snails with a reflected lip at the shell aperture were sampled (hereafter referred to as nonvirgins or N). Snails emerging from hibernation need some time to replenish their energy reserves before they are ready for mating (Baur 2007). The nonvirgins represent snails from different ages and had stored sperm from previous matings (see below). Both virgin and nonvirgin snails were kept isolated in transparent plastic beakers (8 cm depth and 6.5 cm diameter) lined with moist soil (approximately 4 cm) at 19 °C with a light:dark cycle of 16:8 h. Fresh lettuce was provided twice a week and at the same time the beakers were cleaned. Subadult snails terminated shell growth and attained sexual maturity within three weeks. Mated snails usually lay fertilized eggs 3–10 days after copulation (Baur 1988a; Chen and Baur 1993). However, none of these snails produced any eggs indicating that they were virgin. In contrast, all nonvirgin snails deposited fertilized eggs during the three weeks. Thus, three criteria had to be fulfilled to classify a snail as a virgin: (1) It had to be collected prior to the mating season, (2) its shell growth at the time of sampling had not to be completed, and (3) no eggs should be laid when the animal was kept isolated for three weeks. A previous study showed that this procedure is highly accurate to assess virginity in snails collected from natural populations (Kupfernagel and Baur 2011b).

The shell size (breadth and height) of each snail was measured to the nearest 0.1 mm using vernier callipers. Snails were marked individually on their shells with numbers written with a waterproof felt-tipped pen on a

spot of correction fluid. The animals showed no visible reaction to the marking procedure.

One-third of the virgin and one-third of the nonvirgin individuals were randomly assigned to the group of focal snails, while the remaining snails served as potential mating partners in the choice tests. Virgin and nonvirgin snails did not differ in shell breadth (means ± SE, virgins: 17.7 ± 0.1 mm, and nonvirgins: 17.6 ± 0.1 mm, $t_{58} = 0.58$, $p = 0.57$).

Mate-choice tests

Focal snails were allowed to choose between a virgin and a nonvirgin mating partner. For this purpose, each focal snail (virgin or nonvirgin) was placed together with both a virgin and a nonvirgin snail, randomly chosen, in a test arena (transparent plastic container measuring 9 cm × 9 cm × 5 cm). All snails were active at the beginning of the tests, indicated by an extended soft body and everted tentacles. The bottom of the test arena was covered with moist paper toweling to maintain snail activity. Mate-choice tests were performed outdoors at night in May and June 2009 to expose snails to natural changes in light and temperature. This period is the time of maximum mating activity in natural snail populations.

We observed the behavior of each snail at intervals of 15 min using a flash light. For all individuals we recorded six behaviors: (1) withdrawing: the snail was stationary with its softbody retracted; (2) resting: extended softbody, but not moving; (3) crawling: the snail was moving; (4) initial phase of courtship: oral contacts with a potential mating partner, slight eversion of the penial lobe; (5) progressed courtship: close contact to the partner or circling around the partner and eversed genitals; and (6) copulation: simultaneous intromission of the penis of both mating partners. Records also included time until initiation of courtship (courtship latency), courtship duration (from courtship initiation to copulation), and copulation duration. The initiation of courtship was defined as the first simultaneous oral contact (usually accompanied by a slight eversion of the penial lobe in one of the snails) and the beginning of copulation as the first simultaneous intromission. Observation sessions were terminated when a focal snail mated with one of the two partners or after eight hours if no snail initiated courtship behavior. Snails that did not initiate courtship behavior were tested again 10–14 days later in a new set of animals (see below).

The following mate-choice tests were conducted: (1) a virgin focal snail with each a virgin and a nonvirgin snail (test group VVN, the first capital refers to the focal snail, $n = 66$ trials) and (2) a nonvirgin focal snail with each a virgin and a nonvirgin snail (test group NVN, $n = 66$ trials). It is possible that virgin and nonvirgin snails differ in their mating propensity (percentage of individuals mating) under the conditions of our choice tests, which may influence their outcome. To assess the mating propensity of virgin and nonvirgin snails we conducted two additional mating tests: (3) three virgin snails (test group VVV, $n = 32$ trials) and (4) three nonvirgin snails (test group NNN, $n = 32$ trials). The four test groups were equally distributed over 10 nights in which the mating tests were run (altogether 196 trials). A total of 304 snails were used in the trials. 127 (41.8%) were tested once, 91 (29.9%) twice, 65 (21.4%) three times and 21 (6.9%) four times. The majority of copulations occurred in snails tested once.

Sperm number

To determine the number of sperms delivered the focal snails and their mating partners from the test groups VVN and NVN were frozen at −80 °C immediately after the end of copulation. We dissected the female reproductive tract of each snail to obtain the spermatophore received from the partner. In each spermatophore, we measured the area of the outline of the sperm-containing part (A) and its diameter (d) using ImageJ (version 1.43f). Spermatophore volume was approximated assuming the volume of an elongated ellipsoid ($V = (2/3) \times A \times d$).

Sperm counting was performed following the method of Locher and Baur (1997). We mechanically disrupted the spermatophore in 200 µl PBS with Ca^{2+}/Mg^{2+} for 15 min. using microscissors. The spermatophore/sperm suspension was incubated with 1 mg/ml Collagenase/Dispase (Roche, Basel) at 37 °C for 30 min to dissolve tissue and separate sperm. The sperm suspension was then stained with 200 µl of a gallocyanin–chromium complex (a DNA marker). 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 using a microscope (Leica DMLD, magnification 400×) until the total number of sperm heads exceeded 400. The average of the two subsamples was used to calculate the total number of sperm transferred in a spermatophore. The repeatability of this sperm counting method is 0.997 (Locher and Baur 1997). The number of sperms delivered could be assessed in all 24 virgin snails that mated and in 32 of the 36 copulating nonvirgin snails.

Behavioral analyses and statistics

Mating propensity is the percentage of individuals mating. We determined the mating propensity of virgin and nonvirgin snails in the test groups consisting exclusively of virgin (VVV) or nonvirgin (NNN) snails. The mating propensities of virgin and nonvirgin snails were used to correct the expected mating frequencies in the mate-choice tests with the VVN- and NVN-groups (Table 1). Assuming random mating with respect to the mating

Table 1. Results of mate-choice experiments with virgin (V) and nonvirgin (N) individuals of the simultaneous hermaphrodite land snail *A. arbustorum*.

Test group	Number of matings	Focal snail	Mate chosen	Observed number of matings	Expected for random mating[a]	Chi-squared[b]	p	Expected matings considering different mating propensities[c]	Chi-squared[b]	p
VVN	13	V	V	5	6.5	0.69	0.42[d]	5.2	0.01	0.91
			N	8	6.5			7.8		
NVN	17	N	V	6	8.5	1.47	0.23[d]	6.8	0.16	0.69
			N	11	8.5			10.2		

[a]Expected frequencies do not take into account differences in mating propensity.
[b]df = 1 in all cases.
[c]Obtained by weighting the expected frequencies for random pair formation with the mating propensities of virgins and nonvirgins.
[d]Power of the test at $p = 0.05$ is 70.4% for VVN (the experimental design with a power of 80% at $p = 0.05$ would require a 0.777 χ^2 value) and 99.9% for NVN (the experimental design with a power of 80% at $p = 0.05$ would require a 0.679 χ^2 value).

status, then a focal virgin snail has a probability of 50% to mate with either a virgin or a nonvirgin partner in the VVN-test group. The same is true for focal nonvirgin snails in the NVN-test group. We examined deviations between expected and observed numbers of matings using χ^2 tests. Mating trials in which the focal snail was not involved in the copulation were not considered in the analyses.

We illustrated the duration of the different types of behavior (withdrawing, resting, and moving) until one snail initiated courtship in a trial. The mating trials differed in the time elapsed until initiation of courtship. We, therefore, presented the duration of the different types of behavior as percentages. In Figure 1 data of the focal snails and their mating partners were combined and compared with those of nonmating snails. However, the frequencies of different types of behavior of an individual are not independent from each other. We applied Principal Component Analysis (PCA; Legendre and Legendre 1998) to convert the behavior types (withdrawing, resting, moving; see above) of individuals to uncorrelated variables. The first PCA-axis explained well the inter-individual variation in the frequency of the different types of behavior (see results). In further analyses, we used the score of each individual on the first PCA-axis (multiplied by −1) as a measure of activity.

We used linear mixed-effects models (LME; Pinheiro and Gates 2000) with trial as random factor to examine whether mating partners differed in activity from the remaining snail, which did not mate in the same trial and whether mated nonvirgin individuals differed in the degree of activity from mated virgin snails prior to courtship. All statistical analyses were run in the R statistical environment (R Development Core Team 2012), and PCA was performed by the *vegan* package (Oksanen et al. 2011). We calculated the statistical power of χ^2 tests following Cohen (1988) using the *pwr* package of R (Champely 2009). Time until initiation of courtship, courtship duration and sperm number were log-transformed. Means ± 1 SE are given unless otherwise stated.

Results

Mating propensity

Ten successful matings were observed in the 32 trials with three virgin snails (test group VVV), indicating a mating propensity of 31.3%. In the 32 trials with three nonvirgin snails (test group NNN), 15 successful matings were recorded, indicating a mating propensity of 46.9%. Thus, virgin and nonvirgins differed slightly but not significantly in mating propensity ($\chi^2 = 3.28$, df = 1, $p = 0.075$).

Mate-choice tests

Focal virgin snails mated in 13 trials (test group VVN). Nonvirgin individuals were slightly but not significantly more frequently chosen as mating partners than virgin snails (Table 1). When the mating propensities of virgins and nonvirgins were taken into account, the numbers of observed matings with either a virgin or nonvirgin snail were close to the frequencies expected by random mating (Table 1).

Focal nonvirgin snails mated in 17 trials (test group NVN). Again nonvirgin individuals were slightly but not significantly more frequently chosen as mating partners than virgin snails (Table 1). However, the frequencies of virgin and nonvirgin mates did not differ from those predicted by random mating. Considering the slightly different mating propensities, the numbers of observed matings with either a virgin or a nonvirgin snail corresponded to those expected by random mating (Table 1). Thus, neither a virgin nor nonvirgin snails showed any preference for mating with a virgin partner.

Determinants of mating success

In the mate-choice tests, virgin and nonvirgin snails allocated similar proportions of time either to withdrawing, resting or moving (Figure 1(A)). Individuals that eventually mated spent much time moving, while snails which did not mate were mostly resting (Figure 1(B)). Considering exclusively snails that eventually mated, virgin and

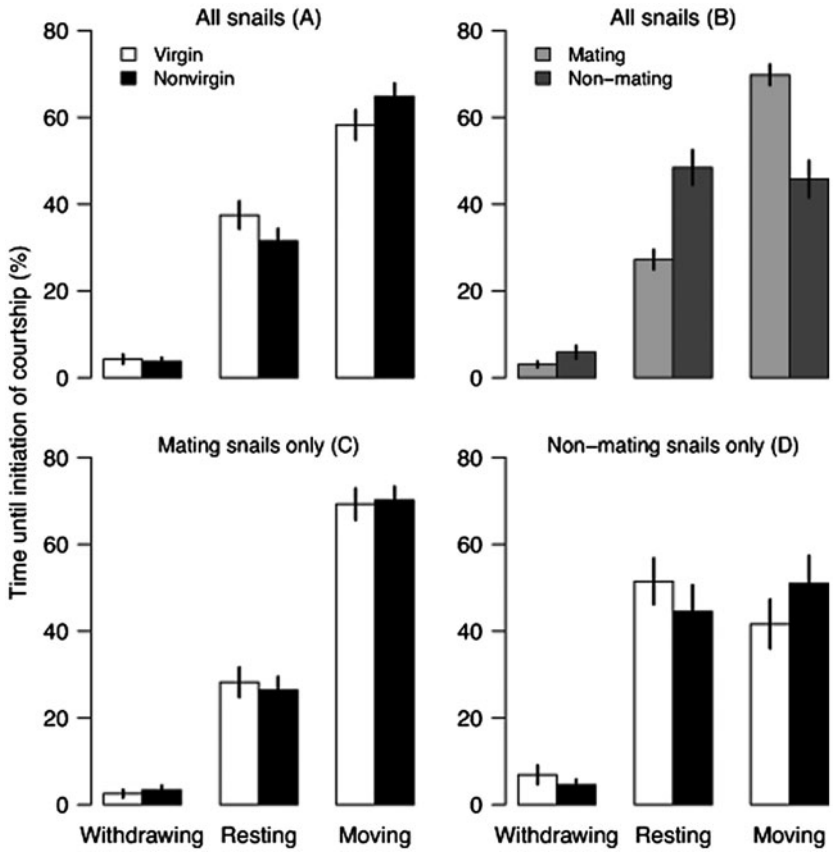

Figure 1. Percentage of time allocated to different behaviors (means ± SE) in virgin and nonvirgin individuals of *A. arbustorum* in mate-choice tests (VNV- and NNV-trials). Comparisons of virgin vs. nonvirgin (A), eventually mating vs. nonmating (B), eventually mating virgin vs. eventually mating nonvirgin (C), and nonmating virgin vs. nonmating nonvirgin snails (D) are shown.

nonvirgin individuals showed similar frequencies in the different types of behavior (Figure 1(C)). Similarly, virgin and nonvirgin snails which did not mate showed similar frequencies of withdrawing, resting, and moving (Figure 1(D)).

PCA was used to convert the nonindependent data on the proportion of different types of behavior to uncorrelated variables for each individual. The interindividual variation in frequency of the different types of behavior could be expressed by the first axis of the PCA, which explained 87.0% of the total variability. Individuals with negative values on the first PCA axis were highly active (spent much time moving), while individuals with positive values on the first PCA-axis were mostly passive (spent much time withdrawing or resting). The second PCA-axis explained the remaining variability (13.0%). We, therefore, expressed the differences in the behavior 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.

Focal snails and partners that eventually mated were significantly more active prior to courtship than individuals that did not mate at all (LME model, $n_{obs} = 210$, $n_{groups} = 70$, $numDF = 1$, $denDF = 139$, $F = 42.16$, $p < 0.001$). Considering the test group VVN, focal virgin snails and their mating partners showed a higher activity before courtship than the remaining snail which did not mate in the same trial (LME model, $n_{obs} = 69$, $n_{groups} = 23$, $numDF = 1$, $denDF = 45$, $F = 17.33$, $p = 0.001$). Similarly, in the test group NVN, the focal nonvirgin snails and their mating partners were more active prior to courtship than the remaining snail (LME model, $n_{obs} = 66$, $n_{groups} = 22$, $numDF = 1$, $denDF = 43$, $F = 15.28$, $p = 0.003$). Mating virgin and nonvirgin focal snails did not differ in activity prior to initiation of courtship (LME model, $n_{obs} = 140$, $n_{groups} = 70$, $numDF = 1$, $denDF = 69$, $F = 0.88$, $p = 0.35$).

Considering exclusively mating focal snails, virgin and nonvirgin individuals differed neither in the time until initiation of courtship (grand mean ± SE = 525 ± 32 min, range: 210–825 min, $t_{28} = 1.71$, $p = 0.097$) nor in the duration of courtship (264 ± 26 min, range: 60–495 min, $t_{28} = 1.79$, $p = 0.084$). Furthermore, the time until initiation of courtship was not influenced

by the mating status of the partner chosen (focal virgin snails: $t_{11} = 0.28$, $p = 0.78$; focal nonvirgin snails: $t_{15} = 1.21$, $p = 0.25$). Similarly, courtship duration was not affected by the mating status of the partner chosen by the focal snails (focal virgin snails: $t_{11} = 0.16$, $p = 0.87$; focal nonvirgin snails: $t_{15} = 1.01$, $p = 0.33$). Copulation lasted 133 ± 12 min in pairs consisting of two virgin snails, 110 ± 6 min in pairs with two nonvirgin snails and was intermediate in pairs consisting of a virgin and a nonvirgin snail (120 ± 8 min). However, copulation duration did not differ statistically between the different types of pair (ANOVA, $F_{2,27} = 1.90$, $p = 0.17$).

Sperm number

In all copulations, spermatophores were transferred reciprocally irrespective of the mating history of donors and recipients. Virgin and nonvirgin snails differed neither in the number of sperm transferred (1248,834 ± 66,121, range: 141,429–2391,625, $t_{54} = 1.20$, $p = 0.236$) nor in the size of the spermatophore delivered (2.11 ± 0.09 mm^3, range: 0.64–3.88 mm^3, $t_{57} = 0.40$, $p = 0.69$). Irrespective of the mating status of the partner (virgin or nonvirgin), snails received similar quantities of sperm (combined data from virgin and nonvirgin sperm recipients ($t_{54} = 0.69$, $p = 0.49$)). Thus, focal snails did not adjust the number of sperm delivered to the mating status of the partner.

Sperm number was positively correlated with the size of the spermatophore delivered ($r = 0.83$, $n = 56$, $p < 0.0001$). However, sperm number was neither correlated with the shell size of the sperm donor ($r = 0.19$, $n = 56$, $p = 0.16$) nor with the size of the recipient ($r = -0.20$, $n = 56$, $p = 0.15$). This suggests that sperm expenditure is not size-related in this simultaneously hermaphroditic snail.

Discussion

The present study revealed that neither virgin nor nonvirgin snails showed any preference for mating with a virgin partner to reduce the risk of sperm competition. Furthermore, virgin and nonvirgin individuals differed neither in number of sperm delivered in the spermatophore nor did they adjust sperm expenditure to the mating status of the partner. The power analysis indicated that the random mating pattern observed can be considered as a reliable finding.

Our mate-choice test aimed to mimic the situation in a natural population in spring when virgin and nonvirgin snails ready to mate encounter each other. Nonvirgin individuals are in general older than virgin ones, have mating experience and may have produced eggs in the preceding year(s). Thus, under natural conditions, virgin and nonvirgin snails not only differ in mating status, but also in other traits. This could influence their mating propensity (see below).

According to theory, mate-choice criteria should vary with the reproductive state, with virgin females being less choosy than mated individuals (Halliday 1983). In our study both focal virgin and focal nonvirgin snails mated randomly with respect to the partners' mating status. Similarly, focal individuals of the basommatophoran freshwater snail *Lymnaea stagnalis* mated randomly with virgin and nonvirgin animals in a choice experiment (Koene et al. 2008). In our study, however, virgin and nonvirgin individuals slightly differed in mating propensity. The mating propensity of snails is partly influenced by the conditions under which the animals are kept and the mate-choice tests are run (Baur et al. 2009). Furthermore, there exist pronounced differences in mating propensity between populations of *A. arbustorum* (Baur and Baur 1992; Baur et al. 2009). Similarly, Fearnley (1996) recorded differences in mating propensity between resident and nonresident individuals of the land snail *Helix aspersa*, which resulted in nonrandom mating in laboratory mate-choice tests. Activity as measured in our study indicates readiness for mating and might also be an indicator for the condition or healthiness of a snail. Most interestingly, activity of the individual *A. arbustorum* recorded over the entire reproductive season was found to be the major determinant of both mating success, and female and male reproductive success (Minoretti et al. 2011). Potential differences in mating propensity have not been considered in other studies on mate-choice preference in simultaneously hermaphroditic gastropods.

In a variety of gonochoristic invertebrate species, growth and size are important indicators for the age and mating status of the potential partner (Thomas 2011). Although shell size is a good indicator of female reproductive output in terms of eggs in *A. arbustorum* (Baur 1988b), this does not result in a preference for mating with larger partners (Baur 1992). In the wild, copulating pairs of *A. arbustorum* consisting either of two individuals which have not yet finished shell growth (subadults) or of one adult (nonvirgin) and one subadult snail have frequently been observed (Baur 1984a; Kupfernagel and Baur 2011a). Subsequent paternity analysis in the offspring of these copulating pairs showed that nonvirgin snails used sperm received from subadult (presumed virgin) mating partners for the fertilization of their eggs in the same frequency as sperm from adult partners (Kupfernagel and Baur 2011a). This indicates that the male function of snails with unfinished growth does not differ from that of adult snails. However, an unfinished shell growth cannot be considered a reliable indicator for virginity in natural populations, because 35% of the subadult snails had already successfully mated prior to the copulation after which they were sampled (Kupfernagel and Baur 2011a). This suggests that neither shell size

nor growth may serve as cue for the mating status of a potential mating partner in *A. arbustorum*. In the present study, the nonexperienced snails used in the test trials were virgins. Otherwise they would have deposited eggs in the laboratory in the 3 weeks prior to the mating trials.

Parker (1983) proposed a model for indiscriminate mate choice (random mating), which should occur when search costs for mates are high (e.g. low encounter rates due to low population densities or low mobility), and/or when there is little variance in mate quality in both sexes. Simultaneously hermaphroditic gastropods show a huge variety of mating behaviors and mechanisms, and it appears to exist that there is no general rule predicting the mating interests of copulating hermaphroditic partners (Baur 1998; Michiels 1998; Jordaens et al. 2005; Jarne et al. 2010; Valdes et al. 2010). Random mating with respect to body size has been reported in the sea hare *Aplysia californica* (Pennings 1991) and in the land snail *Succinea putris* (Jordaens et al. 2005), for shell size and shell colour in the land snail *Cepaea nemoralis* (Wolda 1963), and for shell size and relatedness in *A. arbustorum* (Baur 1992; Baur and Baur 1997). Exceptions from random mating can be explained by physical constraints (e.g. individuals of the marine nudibranch *Chromodoris zebra* that differ greatly in size are unable to bring the reproductive organs together; Crozier 1918) or extended protandric periods, e.g. in the land snail *Achatina fulica* (Tomiyama 1996). In terrestrial gastropod species in which matings are rare, individuals should not be choosy about whom they inseminate.

The lack of pre-copulatory mate choice in *A. arbustorum* can be explained in different ways. It could be a result of the particular biology of the species. In species with low active dispersal like land snails, encounters with potential mates may be rare in low-density populations. Furthermore, sperm depletion over time, most pronounced after hibernation (Baur 1994), may affect the reproductive success. Moreover, courtship and copulation in terrestrial gastropods are restricted to periods of favorable environmental conditions. It has been suggested that because of time-constrained activity and high costs for locomotion, the best strategy for a snail is to mate with the first mating partner available to minimize the risk of either ending up without any mating at all or drying up during mating (Baur 1992). Pre-copulatory selection (mate choice) is therefore regarded as having less impact in land snails, whereas promiscuity and sperm storage promote strong post-copulatory selection (Michiels 1998; Rogers and Chase 2002).

Sperm competition theory predicts that males should allocate sperm according to the number of competing ejaculates. According to the sperm competition risk, a male will invest less sperm when the probability of sperm competition is low and more sperm when the probability is high (Parker et al. 1997). The adaptive significance of increasing sperm number comes from an increased confidence of paternity for males that deliver larger quantities of sperm (Parker 1990a, 1990b). Empirical support for this model has been received in external fertilizing fish species and other taxa including simultaneous hermaphrodites (Velando et al. 2008; Birkhead et al. 2009; Kelly and Jennions 2011). Engqvist and Reinhold (2006) developed models for species with internal fertilization. Their predictions are more complex and depend on the female remating rates and the patterns of sperm utilization. If the remating rate of the recipient is high, Engqvist and Reinhold (2006) predict a higher sperm allocation towards virgins compared to nonvirgins. Our study showed that individuals of *A. arbustorum* do not adjust sperm expenditure to the mating status of the partner, confirming the results of an earlier study in this species (Baur et al. 1998). Similarly, the number of sperm transferred during mating was neither related to the size of the donor nor to the size of the recipient in the land snail *S. putris* (Jordaens et al. 2005).

The random mating pattern and sperm delivery do not necessarily imply random fertilization of eggs. The structure and morphology of the sperm storage site (spermatheca) and fertilization chamber, and the presence of a sperm digestion organ of *A. arbustorum* offer opportunities for sperm selection by the female function of the hermaphrodite (Haase and Baur 1995). For example, Chase and Darbyson (2008) found differential survival of sperm by location within the complexly structured sperm storage organ of the land snail *Cornu aspersum*. Highly skewed paternity patterns in the offspring of mother snails collected in the wild and of snails which copulated with two partners in the laboratory suggest the existence of sperm selection in *A. arbustorum* (Baur 1994; Kupfernagel et al. 2010).

Various mechanisms may influence paternity patterns in simultaneously hermaphroditic gastropods. Individuals of many stylommatophoran species use a dart to pierce the body of the mating partner during courtship (Chase 2007). The substance adhering to the dart induces conformational changes in the female reproductive tract of the recipient, closing off the entrance to the gametolytic bursa copulatrix, and thus most likely reduce sperm digestion in *H. aspersa* (Chase and Blanchard 2006). Successful dart shooting, doubles the number of donated sperm that are stored by the recipient and it significantly increases the relative paternity when a dart shooter competes with an unsuccessful shooter (Rodgers and Chase 2002). Furthermore, the length of the spermatophore producing organ (epiphallus) may have an influence on the number of allosperm that reach the storage organ in *H. aspersa* and *Euhadra peliomphala* (Garefalaki et al. 2010; Kimura and Chiba 2013). Longer spermatophores require more time to reach the gametolitic organ and

begin to be digested (Kimura and Chiba 2013). A delay in spermatophore digestion could explain the high fertilization success shown by snails with long spermatophores.

The lack of mate choice with respect to mating status does not imply that there is no pre-copulatory mate choice at all in *A. arbustorum*. Previous choice experiments revealed pre-copulatory discrimination between individuals from two distant populations of *A. arbustorum* in Switzerland, suggesting incipient reproductive isolation (Baur and Baur 1992; Kupfernagel and Baur 2011b). It is assumed that individuals may use population-specific cues (pheromones and behavior) during courtship and copulation to assess the affiliation of potential mates.

Acknowledgments

We thank L. Schärer for advice and two anonymous reviewers for their valuable comments on the manuscript. Financial support was received from the Swiss National Science Foundation.

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General Discussion

Various factors may impact sperm traits and reproductive strategies in simultaneously hermaphroditic gastropods. While many factors contribute to sperm traits, a parasitic infestation has a major impact on life-history and therefore reproduction of the host. The main focus of this thesis was to investigate the effects of selected life-history traits, morphological characteristics, habitat conditions and especially parasitic infestation on sperm size.

In **Chapter I**, I examined the hibernation process of the hematophagous mite *R. limacum* to estimate the impact on the survival of its host *A. arbustorum* and to find out why these parasitic mites do not occur in host populations at elevations of 1290 m or higher. One hypothesis was that the hibernation period of the host might be too long at high elevations for mites and their eggs to survive. To test this hypothesis, we experimentally infected snails and allowed them to hibernate in a climate chamber at 4 °C for periods of 4, 5, 6 and 7 months. The host snails' winter survival was negatively affected by parasite infection, but not by duration of hibernation. On the other hand, the intensity of mite infection decreased with increasing hibernation duration. A short-time experiment with 0 (control), 4, 8, 12 days of hibernation and shorter recording intervals revealed that mites do not leave the host when it buries in the soil at the beginning of hibernation. In this experiment, the number of mites and eggs found in the lung tissue during dissection did not differ. Only after 24 days of hibernation the number of mites started to decrease, whereas the number of eggs remained constant throughout hibernation. Consequently, *R. limacum* survives hibernation in the egg stage attached to the lung tissue of *A. arbustorum*. Results of a third experiment indicated that mite survival in the soil was very low during winter.

In summary, these results suggest that low temperature at high elevations limited the occurrence of *R. limacum.* Former studies on a parasitic nematode and its host snails showed that temperature was a limiting factor for parasite development (Halvorsen & Skorping 1982, Schjetlein & Skorping 1995). Furthermore, infected populations of *A. arbustorum* were located at sites in Switzerland with a higher mean temperature in June compared to uninfected populations (Baur & Baur 2005). Although the minimum temperature for its development is not known, the results indicated that *R. limacum* prefers moderate climate conditions.

A reduced winter survival of the host confirms the findings of former studies on naturally infected individuals of *Arianta arbustorum* (Schüpbach & Baur 2008, 2010).

The aim of **Chapter II** was to investigate geographical variation in sperm morphology over the distribution range of the land snail species (*Arianta arbustorum*) in relation to infection with its parasite (*Riccardoella limacum*).

The results showed a significant variation in total sperm length among the 23 populations studied, with a difference of 11 % of total sperm length between the shortest and longest population means. Mantel tests identified significantly increased inter-population differences in total sperm length with increasing geographical distance between populations. A minimal adequate model revealed that parasitic infection had a positive effect on total sperm length. Infected individuals of *A. arbustorum* produced larger sperm than uninfected snails, independent of the population examined. Additionally, longitude had a negative effect on sperm length, as it decreased from West to East. In a subsample of 12 populations restricted to the mountains of Switzerland (elevation range: 440–2485 m a.s.l.), total sperm length decreased with increasing elevation. In populations above an elevation of 1500 m, no infected snail could be detected (see also Baur & Baur 2005). The analysis of sperm head length also showed a variation among populations but this was not influenced by any of the factors assessed in the studies presented here.

These results confirmed that diverse selection pressures might have an impact on sperm length. However, selection pressures acting among population seem to differ from those acting within. An infection with parasites reduces fitness and survival (Schüpbach and Baur 2008, Wacker 2008, Jaenike et al. 1995) and could therefore cause an increase of reproductive effort (Polak & Starmer 1998). The influence of *R. limacum* on male investment of *A. arbustorum* has not been studied to date. The larger sperm produced by infected snails indicates a parasite-induced alteration of sex allocation by an increased investment in the male function and thus conforms to the theory of sex allocation in hermaphrodites (Charnov 1996). Thus, decreased sperm length in alpine populations could be explained by the absence of the parasite. Furthermore, shortage of nitrogen in high altitudes (Baur & Baur 1998) and reduced intensity of sperm competition (Beese et al. 2006) could result in shorter spermatozoa. In *A. arbustorum,* researchers found a genetic determination of sperm length. Total sperm length did not differ in sperm delivered by the same individuals in 2–4 matings over two reproduction seasons (Minoretti et al. 2013). Hence, the reduced variation observed in sperm length among individuals within populations might be a result of stabilizing selection.

The main focus of chapter **Chapter III** was to test the hypothesis that self-fertilizing hermaphroditic species produce shorter sperm due to the fact that there is no other mating partner and thus there is no sperm competition, in contrast to cross-fertilizing species, which use sperm stored from several mating partners for the fertilization of their eggs and thus are exposed to intense sperm competition (Baur 1998, Kupfernagel et al. 2010). Theoretical models predict that sperm competition could favour the evolution of longer sperm (Parker 1993). To examine interspecific variation in sperm traits in relation to different breeding systems, I measured sperm length and sperm head length of 57 species of simultaneously hermaphroditic stylommatophoran gastropods from Europe and South America. 28S rRNA, nuclear and COI mitochondrial sequence data were used to construct a molecular phylogeny. Phylogenetic generalized linear models were applied to examine the potential influence of morphological and life-history characters. Species with cross-fertilization had longer sperm than species with self-fertilization or a mixed breeding system. The best-fit model revealed that sperm length and sperm head length were influenced by the breeding system, gastropod size and shell type. Additionally, sperm length and sperm head length increased with the size of the species.

Results of decades of research on sperm biology suggested that sperm size might be influenced by sperm competition and the fertilization environment (the female reproductive tract) (reviewed in Pitnick et al. 2009). Sperm size may influence swimming speed (Gomendio & Roldan 2008) and larger sperm might have better chances to reach the storage organs. Furthermore, larger spermatozoa, stored in the spermatheca, could displace sperm from subsequent mates with the beating of their long flagella (Rogers & Chase 2002). A recent study found a correlation of morphological evolution between sperm length and carrefour length (spermatheca and fertilization pouch) in stylommatophoran gastropods (Beese et al. 2009). Concerning the effects of morphology on sperm traits, findings of a positive relationship between sperm length and shell size supported the results of Beese et al. (2009) and extended knowledge of the positive sperm length–body size relationships in other invertebrates (reviewed by Pitnick et al. 2009).

The aim of **Chapter IV** was to investigate the influence of mating status and, therefore, sperm competition on mating behaviour and to find out if ejaculate adjustment (sperm number) could be a consequence. In a species with multiple mating and sperm storage, males

are expected to show a preference for mating with virgin and young females to reduce the risk of sperm competition.

In all trials the observed mating was random, which means neither virgin nor nonvirgin snails showed any preference for mating with a virgin partner. The mating success was mainly dependent on the activity of the individual. Focal snails and partners that eventually mated were significantly more active prior to courtship than individuals that did not mate at all. The mating status of the partner chosen did not have any influence on the time until initiation of courtship, duration of courtship, or on copulation duration. Furthermore, snails in all test situations did not adjust the sperm number delivered to the mating status of the partner. Sperm competition theory predicts that males should allocate sperm according to the number of competing ejaculates. According to the sperm competition risk, a male will invest less sperm when the probability of sperm competition is low and more sperm when the probability is high (Parker et al. 1997). In contrast, Engqvist and Reinhold (2006) predict a higher sperm allocation towards virgin mates compared to nonvirgin mates, if the remating rate of the recipient is high. However, results of this investigation did not detect any adjustment of sperm investment to the mating status of the partner in *A. arbustorum*, confirming the results of a former study (Baur et al. 1998).

According to theory, mate-choice criteria should vary with the reproductive state, with virgin females being less choosy than mated individuals (Halliday 1983). However, random mating in relation to mating status was also found in the freshwater snail *Lymnaea stagnalis* (Koene et al. 2008). Furthermore, different snail species mate randomly with respect to other traits such as body size, shell size, shell color and relatedness of populations (Pennings 1991, Jordaens et al. 2005, Wolda 1963, Baur 1992, Baur and Baur 1997). Random mating might occur when the costs to search for a mate are high and/or when there is little variance in mate quality in both sexes (Parker 1983).

Activity could be seen as an indicator for overall condition, healthiness or readiness for mating. In a study on *Arianta arbustorum* with records over the entire reproductive season, activity was found to be the major determinant of both mating success, and female and male reproductive success (Minoretti et al. 2011).

Random mating itself does not imply a random fertilization of eggs. The presence of a sperm-digesting organ and the morphology of the sperm storage organ allow a selective storage and use of sperm in *A. arbustorum*. Additionally, highly skewed paternity patterns in the offspring of mother snails collected in the wild and of snails that copulated with two partners in the laboratory suggest the existence of sperm selection in *A. arbustorum* (Baur

1994, Kupfernagel et al. 2010). The lack of pre-copulatory mate choice in *A. arbustorum* could be explained as follows. In species with low active dispersal and possible low-density populations it is difficult to find potential mates. Furthermore, it has been suggested that time-constrained activity and high costs for locomotion lead to the strategy to mate with the first mating partner available to minimize the risk of either ending up without any mating at all or drying up during mating (Baur 1992). Pre-copulatory selection (mate choice) is therefore regarded as having less impact in land snails, whereas promiscuity and sperm storage promote strong post-copulatory selection (Michiels 1998, Rogers & Chase 2002).

OUTLOOK

Reproduction in simultaneously hermaphrodites is driven by complex mechanisms, which are not yet fully understood. This thesis provides new insights into the reproduction of Stylommatophora, especially the land snail *Arianta arbustorum*. Parasite-host dynamics of *Riccardoella limacum* and helicid snails were intensively investigated in former studies (Baker 1970, Schüpbach & Baur 2008, 2010), however, information about the impact of the parasite on host's reproduction is still lacking. Results presented in this thesis revealed that infection with the parasite *R. limacum* had a positive effect on sperm length of *A. arbustorum*. It is an open question which mechanisms could be responsible for this effect. Additionally, it could be demonstrated that *R. limacum* is sensitive to low temperatures over a longer period (>24 days), consequently to longer hibernation periods. This supported former observations that this parasite does not occur in alpine host populations on elevations over 1290 m (Baur & Baur 2005). At lower elevations, the parasitic mite seems to survive in the egg stage, although further studies have to be conducted to complete information about egg survival, egg development and development temperatures of *R. limacum*. Furthermore, an interesting question is whether climate warming with increased mean annual temperatures and decreased hibernation durations influences the host-parasite relationships. A rise in the temperature for an extended period of time might, therefore, have an impact on the snail populations at high elevations.

One focus of this thesis was to shed light to selection pressures affecting sperm traits in stylommatophoran species. Self-fertilizing species were expected to have shorter spermatozoa due to the absence of sperm competition. The results showed that the breeding system and therefore sperm competition risk affect sperm length and thus supported this

hypothesis. Cross-fertilizing species had longer sperm than self-fertilizing species and species with mixed breeding. However, if cross-fertilization selects for longer sperm, a mixed mating system was predicted to show a more similar sperm morphology to cross-fertilizing species. Available information indicates that species with a mixed breeding system fertilize 70–80% of their eggs by self-fertilization, suggesting less intense sperm competition than in cross-fertilizing species. This might be an explanation for the small difference in sperm length between species with predominant self-fertilization and species with a mixed breeding system.

To summarize, many aspects of sperm traits in stylommatophoran snail species are still not fully understood. Furthermore, global climate change and rising temperatures may have an impact on snail populations that are partly found in geographically isolated areas. A deeper understanding of the influence of environmental factors on sperm traits may elucidate the risks or benefits of a rapidly changing environment on sperm traits of other species. Therefore, this research provided a foundation for future studies on sperm traits and emphasizes the need for profound knowledge in this subject area.

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