THE EVOLUTION OF MALE AND FEMALE REPRODUCTIVE TRAITS IN SIMULTANEOUSLY HERMAPHRODITIC TERRESTRIAL GASTROPODS

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Kathleen Beese

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Prof. Dr. Bruno Baur
PD Dr. Andreas Erhardt

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Prof. Dr. Hans-Peter Hauri
Dekan

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SUMMARY

Our understanding of postcopulatory sexual selection forcing reproductive trait evolution continues to be illuminated by comparative studies. Inter- as well as intraspecific comparisons offer the opportunity to study the long-lasting processes of diversification and allow testing for correlated evolution between different traits. Moreover, morphological studies provide important insights into the function and adaptive significance of specialised reproductive organs.

In this thesis, I combined comparative studies on the inter- and intraspecific evolution of female sperm storage organs (spermathecae) and sperm traits in stylommatophoran gastropods with detailed studies on the influence of spermatheca morphology on sperm storage patterns and the adaptive function of the bursa tract diverticulum, an organ of the reproductive tract of snails associated with sperm digestion, in the helicid land snail *Arianta arbustorum*.

In order to assess the pattern of sperm storage organ divergence across 47 species of stylommatophoran snails and slugs partial 28S rDNA sequences were used to construct a molecular phylogeny. Maximum likelihood as well as Bayesian methods were applied to investigate the history of spermatheca origination and to test different hypotheses of spermatheca evolution. The results revealed a large variation in the presence/absence of a spermatheca and its structural complexity across stylommatophoran gastropods. The evolution of spermathecae in the carrefour appeared to be associated with the evolution of other peculiar morphological traits of the reproductive tract, e.g. love-dart shooting, as well as with flagellum and diverticulum length. Moreover, a close relationship of spermatheca presence with cross-fertilization as the predominant mating system was found. In addition, the presence of complex spermathecae was coupled with several life-history traits, including body size, reproductive strategy (semelparity vs. iteroparity) and reproductive mode (oviparity vs. ovoviviparity), and with habitat specificity. Sperm length, highly diverse in this species group, appeared to be adapted to the length of the sperm storage organ. The results suggest an important influence of postcopulatory sexual selection on spermatheca divergence. However, also life-history traits and habitat specificity might have shaped the pattern of spermatheca distribution found across stylommatophoran gastropods.

A closer look at male and female reproductive trait divergence, focussing on sperm traits and sperm storage organ size, was taken using six natural populations of *Arianta arbustorum*. The intraspecific variation in spermatophore volume, number of sperm transferred and sperm length as well as in volume and length of the spermatheca and the number of sperm storage tubules was quantified and the covariation between interacting traits was examined. A significant among-population variation was revealed for all traits except for spermatheca length. Furthermore, a positive association was found between the number of sperm transferred and spermatheca volume. In accordance with the interspecific study, these results indicate a strong influence of antagonistic coevolution on male and female reproductive trait evolution.

Beside size and morphology of sperm storage organs, the physical properties of the spermatheca may be important for the potential to exert cryptic female choice. This was investigated by examining structure, volume and tubule length of empty spermathecae of *A. arbustorum* and assessing differences in spermatheca size following a single copulation. The study revealed that spermathecae of this species are expandable and can accommodate more sperm than would be expected from measuring its initial volume. Moreover, neither the volume of sperm stored in the spermatheca nor the amount of allosperm digested in the bursa copulatrix were related to the size of the spermatophore received. These findings suggest that the female function may be able to control sperm storage and sperm use.

Finally, the morphology and function of the bursa tract diverticulum, which serves as a place of spermatophore uptake when present, was studied. Using histological, histochemical and morphometrical methods it could be shown that the diverticulum is involved in the digestion or at least in the partly breakdown of received spermatophores. Furthermore, the positive allometry and the high phenotypic variation of diverticulum length compared to shell size suggest directional sexual selection on this trait. Combining evidence from this and previous studies indicates that the diverticulum is involved in the coevolution of the complex reproductive traits of stylommatophoran gastropods.

GENERAL INTRODUCTION

SEXUAL SELECTION AND SEXUAL CONFLICT

Sexual selection, defined as "selection that arises from differences in mating success" (Darwin, 1871), is a fundamental component of evolutionary biology. This fact has already been recognized by Darwin (1871) in his famous book *The descent of man and selection in relation to sex*. However, for a long time, under the influence of Darwin's work, sexual selection has been seen to occur exclusively "before parents unite", because Darwin assumed females to be sexually monogamous. Only in the past 30 years it has become apparent that females are far from monogamous, and recently it has been shown that not only males but also females can gain from polyandry (Jennions & Petrie, 2000). Polyandry has important biological implications because sexual selection can continue even after insemination. Postcopulatory sexual selection comprises both postcopulatory male-male competition, a phenomenon described as "sperm competition" (Parker, 1970), and female controlled processes that bias the fertilization success of the males that copulate with and inseminate them termed "cryptic female choice" (Eberhard, 1996).

Furthermore, in contrast to the traditional view of reproduction as being a largely harmonious event in which males and females cooperate in producing offspring, intense postinsemination sexual selection can create the potential for sexual conflicts, broadly defined as "differences in the evolutionary interests between males and females" (Parker, 1979). Such differences are created by the differential investment made by males and females in reproduction, which is usually predicted by anisogamy (Chapman, 2006). Whenever the different optima for males and females cannot simultaneously be realised, there will be sexual conflict. The importance of sexual conflict is that it has the potential to drive rapid evolutionary changes via sexually antagonistic coevolution, generating a startling diversity of behavioural, physiological and morphological adaptations (Birkhead & Pizzari, 2002).

One major part of studies on postcopulatory sexual selection focussed on the evolution of sperm traits under the influence of sperm competition (Birkhead & Møller, 1998). However, in internally fertilizing taxa the competition between sperm of different males takes place within the female reproductive tract. Therefore, not only sperm traits but also female reproductive traits, which may influence the pattern of sperm storage and use

are central to the fertilization process. In many taxa, the female reproductive morphology is highly complex and models of sperm storage suggest that females with multiple storage sites have much greater flexibility to control or influence offspring paternity by postcopulatory sperm selection (Hellriegel & Ward, 1998). Moreover, variation in size, shape and physical properties of female sperm storage organs have been related to the possibility to exert cryptic female choice (Walker, 1980; Simmons, 2001).

The interaction of male ejaculate characteristics with the morphological and physiological environment of the female reproductive tract may generate correlated evolutionary changes and likely contributes to reproductive isolation among populations and eventually to the formation of new species (Parker & Partridge, 1998). A pattern of coevolution between sperm traits and several characteristics of the female reproductive tract has been revealed by comparative studies on a diverse array of taxa (e.g. Dybas & Dybas, 1981; Briskie & Montgomerie, 1992; Pitnick et al., 1999; Presgraves et al., 1999; Minder et al., 2005).

POSTMATING CONFLICT IN HERMAPHRODITES

Although hermaphroditism is widespread in the animal kingdom, occurring in very different groups such as opisthobranch sea slugs, pulmonate snails, some crustaceans, flatworms, earthworms, leeches and arrow-worms, sexual selection in this form of gender expression is just starting to receive increased attention from evolutionary biologists (Michiels, 1998; Schilthuizen, 2005). Simultaneous hermaphrodites differ from species with separate sexes (gonochorists) in that they possess both functional male and female reproductive organs at the same time and that reproductive acts usually involve male and female function in each individual. Because of this condition, Darwin (1871) believed that sexual selection could not act in hermaphroditic organisms; and although attempts have been made to understand selective forces in hermaphrodites (Charnov, 1979) until recently it was expected that the opportunity for sexual selection is at most half that of separate-sex taxa (Greeff & Michiels, 1999a). In opposition to this, new models suggest that simultaneous hermaphrodites inherently are more prone to be caught in costly escalations than gonochorists, mainly because within one mating simultaneous hermaphrodites gain paternity (male fitness) which can outweigh the loss in female fitness (Michiels & Koene, 2006).

Especially internally fertilizing hermaphrodites often have strikingly complex reproductive morphologies and mating frequently involves overt aggression or injurious mechanisms (Michiels, 1999). Examples of bizarre behaviours and structures include the penis chewing of *Ariolimax* (Leonard et al., 2002), the repeated hypodermic insemination in tropical flatworms (Michiels & Newman, 1998) and the injection of allohormones (Koene & ter Maat, 2001) into the body of the mating partner via copulatory setae in *Lumbricus terrestris* (Koene et al., 2002; Koene et al., 2005) or via love darts in land snails (e.g. Chase & Blanchard, 2006). All these structures are assumed to manipulate the mating partner in favour of the own reproductive success and therefore may provoke counteradaptations by the sperm receiver, leading to cycles of antagonistic evolution of reproductive traits (Rice & Holland, 1997) or to the evolution of new traits (Lessells, 2006).

REPRODUCTIVE MORPHOLOGIES IN STYLOMMATOPHORAN GASTROPODS

The large group of stylommatophoran gastropods offers an exceptional opportunity to study reproductive trait evolution in simultaneously hermaphroditic animals. Snails and slugs show a wide diversity of reproductive characters and the mating systems ranges from exclusive self-fertilization to obligate cross-fertilization. In many stylommatophoran species, multiple mating with different mating partners, long-term sperm storage and sperm digestion are common (Baur, 1998). Nevertheless, detailed studies on the evolution of complex male and female reproductive traits are scarce and deal mainly with particularly striking structures and behaviours such as love dart shooting (Davison et al., 2005; Koene & Schulenburg, 2005). Such comparative studies revealed a pattern of covariation between several reproductive measures, which suggests that sexual selection and sexually antagonistic coevolution are important factors in their evolution (Schilthuizen, 2005).

The female reproductive tract often harbours complex organs, which might allow for cryptic female choice via selective sperm storage and use or via sperm digestion (Baur, 1998). Stylommatophoran gastropods show an enormous inter- and intraspecific variation in the structure of the spermatheca (Baur, 1998). In some species, this sperm storage organ consists of many spermathecal tubules and thus the female reproductive system may be able to control fertilization by a spatial separation of sperm from different mating partners (Haase & Baur, 1995). Nevertheless, studies on the variation in spermatheca morphology

and patterns of sperm storage exist only for *Arianta arbustorum* and *Helix aspersa*, both helicid land snails (Baminger & Haase, 1999; Rogers & Chase, 2001, 2002; Bojat & Haase, 2002; Evanno et al., 2005).

Moreover, although important for postcopulatory sexual selection, sperm traits have been intensively studied in stylommatophoran gastropods only with regard to interspecific variation in sperm length (Thompson, 1973). It has been found that sperm of many stylommatophoran gastropods are considerably long, exceeding 1000 μm in some species. In addition, a recent study in *A. arbustorum* revealed a high intraspecific variation in sperm length (Minoretti & Baur, 2006). However, the variation of sperm number within and across species remains largely unknown. The only study on this topic found a significant difference in the amount of sperm transferred across four population of *A. arbustorum* (Minoretti & Baur, 2006).

Sperm digestion, which occurs in the bursa copulatrix of stylommatophoran snails and slugs (Németh & Kovács, 1972; Rogers et al., 1980; Gomez et al., 1991), reduces the competitive ability of an ejaculate (Greeff & Michiels, 1999b). Therefore, Greeff & Michiels (1999b) suggested that sperm digestion coupled with sperm competition should lead to an intersexual arms race, with the male component evolving to transfer larger ejaculates and the female component evolving to digest more sperm. Thus, it can be expected that reproductive organs associated with sperm production, sperm transfer as well as sperm digestion are caught in cycles of antagonistic coevolution, leading to an increased diversity of reproductive morphologies. In accordance with this, Koene and Schulenburg (2005) found in a comparative study indications for a correlated evolution between organs producing the spermatophore's tail and spermatophore receiving organs.

OUTLINE OF THE THESIS

This thesis consists of four major chapters that can be read independently. Different aspects of the evolution of male and female reproductive morphologies in stylommatophoran gastropods are considered. The studies presented here aim to establish a relationship between the highly complex reproductive tract of snails and slugs with the different postcopulatory selection pressures influencing their divergence, including cryptic female choice, sperm competition and sexual conflict.

Chapter I comprises the results of a phylogenetic comparative study on the distribution and evolutionary history of spermatheca presence and complexity in the carrefour of 47 species of stylommatophoran gastropods. The study tested different hypotheses of sperm storage organ evolution by examining potential associations of spermatheca presence and complexity with the presence of other reproductive characters and also with several life-history traits and with habitat specificity. Moreover, it was investigated whether spermatheca size and sperm size are coevolving.

In **Chapter II**, the intraspecific variation in male and female reproductive traits across six natural populations of the land snail *Arianta arbustorum* was studied. In particular, the covariation in interacting traits was examined to determine whether the variation in spermathophore volume, number of sperm transferred and sperm length as male traits and spermatheca length, spermatheca volume and number of sperm-storing tubules as female traits results from divergent coevolution.

To gain a better knowledge of sperm storage processes, the physical properties of the female sperm storage organ, including its initial size and sperm storage capacity, were investigated in *A. arbustorum*. The volume and tubule length of empty spermathecae were examined in relation to the variable spermatheca structure and changes in spermatheca size after sperm uptake were assessed. Furthermore, the influence of copulation duration on the amount of sperm stored and the relationship between sperm transfer and sperm use were assessed. The results are presented in **Chapter III**.

The major aim of **Chapter IV** was to investigate the adaptive function of the bursa tract diverticulum, a reproductive organ that occurs in many helicid snail species as an appendix of the sperm digesting bursa copulatrix. Using *A. arbustorum* as a model species, the ultrastructure of the diverticulum is described for the first time. Moreover, structural changes of this organ after mating were analysed using morphometric, histological as well as histochemical methods. The duration of spermatophore presence and sperm survival were assessed and the interindividual variation in diverticulum length in relation to shell size was evaluated. Finally, Chapter IV proposes a hypothesis for the evolution of spermatophore-receiving structures in helicid land snails.

CHAPTER I

EVOLUTION OF FEMALE SPERM STORAGE ORGANS IN THE CARREFOUR OF STYLOMMATOPHORAN GASTROPODS

Kathleen Beese, Georg F. J. Armbruster, Konstantin Beier & Bruno Baur

ABSTRACT

The presence of specialized female sperm storage organs has been widely recognized as an important factor influencing postcopulatory sexual selection via sperm competition and cryptic female choice in internally fertilizing species. We morphologically examined the complexity of spermathecae in the carrefour in 47 species of stylommatophoran gastropods, a large hermaphroditic group that exhibits an extraordinary diversity of reproductive characters. We used partial 28S rDNA sequences to construct a molecular phylogeny for these species, and applied maximum likelihood and Bayesian methods to investigate the history of spermatheca diversification and to test different hypotheses of sperm storage organ evolution. Our phylogenetic analyses revealed several independent gains and losses of spermathecae in stylommatophoran gastropods indicating rapid evolutionary changes. Moreover, consistent with the theory that postcopulatory selection is a strong force in shaping reproductive morphology, a complex spermatheca was associated with the occurrence of love darts or any kind of auxiliary copulatory organ, the presence of a long flagellum and cross-fertilization as the predominant mating system. However, our results also suggest associations of carrefour complexity with body size, reproductive strategy (semelparity vs. iteroparity), reproductive mode (oviparity vs. ovoviviparity), and with habitat type. We also measured sperm and carrefour length in 17 snail species possessing a spermatheca. There was a positive correlation between carrefour length and sperm length. Our findings indicate that different factors influence the evolution of female sperm storage organs in hermaphroditic gastropods. It remains a challenge to disentangle the effects of sexual selection, life history, and habitat specificity on reproductive trait divergence in this animal group.

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INTRODUCTION

In the majority of internally fertilizing animals the processes of insemination and fertilization are uncoupled in space and time. In these species, sperm are preserved in the reproductive tract of the female for weeks, months or even years before being used to fertilize eggs (Birkhead & Møller, 1998). The ability to store sperm is an integral part of the species' reproductive strategy and can provide important advantages (Neubaum & Wolfner, 1999). However, despite recent studies demonstrating that females of many taxa possess highly complex organs for storage of sperm (e.g., insects: Pitnick et al. 1999; crustaceans: Bauer and Martin 1991; reptiles: Olsson and Madsen 1998, and birds: Shugart 1988), the causes of interspecific divergence in the presence and morphology of these organs are still not well understood, particularly in hermaphroditic animal species.

A variety of adaptive explanations have been proposed to explain the diversity of female sperm storage organs. One widely supported hypothesis is that postcopulatory sexual selection has played an important role in the evolution of this trait, due to the potential influence of female sperm stores on the extent of non-random paternity (Eberhard, 1996; Pitnick et al., 1999). A prerequisite for sexual selection via sperm competition is that the sperm of two or more males coexist within the reproductive tract of the female at the time of fertilization (Parker, 1970). In the past few years, increasing attention has been paid to the possibility that females of many species are active not only in precopulatory choice but also in controlling the processes of sperm storage and use (Eberhard, 1996; Birkhead & Pizzari, 2002). The presence of storage organs may allow females to maintain viable sperm from multiple mates and thus selectively bias the fertilization success of sperm in relation to male behavior (Siva-Jothy & Hooper, 1995) or male genotype (Ward, 1998a). Males always try to monopolize females (Chapman et al., 2003). Females, however, may benefit from increased within- and between-male variance in sperm traits in their reproductive tract (Jennions & Petrie, 2000). The resulting male-female conflict over sperm use could have favored the evolution of adaptations in the female that control the events after copula and, vice versa, counter-adaptations by the male that manipulate sperm storage processes (Parker, 1979; Rice & Holland, 1997). These adaptations often involve harmful behavior and might lead to perpetual antagonistic co-evolution between certain traits (Rice & Holland, 1997) or the evolution of new traits (Lessells, 2006), resulting in increased inter-sexual specializations. The presence of female

sperm storage organs should therefore be linked with the presence of complex or peculiar reproductive traits. Moreover, diverse mating systems that impose different levels of selection pressure on postcopulatory processes are expected to covary with the presence of sperm storage organs and their complexity.

Other hypotheses claim that the differentiation of female sperm storage organs is dictated by demands of sperm storage capacity arising from differences in animal longevity and/or egg productivity, or by selection for functional design to match sperm morphology in order to efficiently store and utilize sperm (Pitnick et al., 1999). Females with a high longevity or producing multiple egg clutches in consecutive years may require more specialized organs to provide nourishment or protection (e.g., through anchoring the sperm inside the storage organ) to maintain the viability of sperm (Tingari & Lake, 1973; Smith & Yanagimachi, 1990). Consequently, the evolution of sperm storage organs should be coupled with life history. Moreover, female reproductive morphology is presumably associated with habitat specificity, because of adaptations of the life-history traits to local conditions. The evolution of female morphology may also simply track sperm length that evolves due to selection independent of female sperm stores (Pitnick et al., 1999), which might result in evolutionary correlations between the length of sperm storage organs and sperm length documented in several studies (Dybas & Dybas, 1981; Briskie & Montgomerie, 1992; Presgraves et al., 1999).

The large group of terrestrial snails and slugs (Stylommatophora) is an excellent model system to investigate the evolution of female sperm storage organs in hermaphroditic animals. Snails and slugs show a wide diversity of reproductive characters, including the stabbing of love-darts into the mating partner during courtship and organs for the digestion of received sperm (=allosperm), and a mating system that ranges from almost exclusive self-fertilization to obligate outcrossing. Furthermore, snails and slugs exhibit a great variety of life-history characters and habitat specificities. Moreover, sperm of gastropods are considerably long, exceeding 1000 μm in length in some species (e.g., 1750 μm in *Pleurodonte acuta*; Thompson 1973).

Postcopulatory sexual selection may have had an important influence on the evolution of the complex and bizarre reproductive characters (Schilthuizen, 2005). However, the rare comparative studies in snails and slugs have mainly focused on particularly striking structures and behaviors, e.g., love-dart shooting (Davison et al., 2005; Koene & Schulenburg, 2005). In contrast, our knowledge of female sperm storage is still insufficient. Previous studies have shown that in some species of stylommatophoran

gastropods the storage of allosperm occurs in the spermatheca, a portion of the carrefour (van Mol 1971; Haase and Baur 1995). An illustration of the reproductive tract with the carrefour is presented in Figure 1. The spermatheca can be subdivided into multiple tubules, and in this case a spatial separation of sperm stored from different mates and thus presumably female control of paternity could be possible (Hellriegel & Ward, 1998). However, in other species no spermatheca is found in the carrefour (Kugler, 1965; Els, 1974).

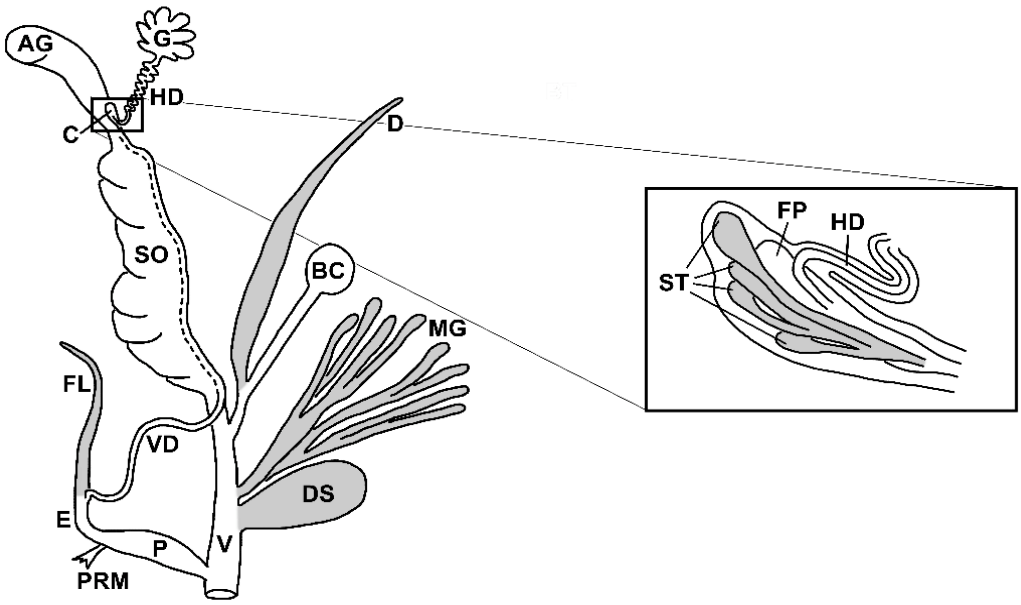

Figure 1. Schematic representation of the reproductive tract and the carrefour (inset) of stylommatophoran gastropods. The structures in gray (DS, dart sac; MG, mucous glands; FL, flagellum and D, diverticulum) are not present in all species. The carrefour (C) in the proximal genital system consists of a fertilization pouch (FP) and may additionally possess a spermatheca with one or multiple sperm storing tubules (ST). Other abbreviations: AG, albumen gland; BC, bursa copulatrix; E, epiphallus; G, gonads; HD, hermaphroditic duct; P, penis; PRM, penis retractor muscle; SO, spermoviduct; V, vagina; VD, vas deferens.

In the present study, we examined the pattern of spermatheca evolution in the carrefour of stylommatophoran snails and slugs using a comparative phylogenetic approach. To offer a more rigorous picture of character evolution, we established the relationships among the species and explored the character histories using maximum likelihood and Bayesian inference. We used character association statistics to analyze three mutually not exclusive hypotheses of female sperm storage organ evolution: (1) spermatheca presence and complexity is a consequence of postcopulatory sexual selection and is therefore coevolving with other reproductive characters; (2) differences in life

history and/or habitat specificity are important for carrefour diversification; and (3) the evolution of carrefour size is dictated by utilitarian demands of efficient sperm storage. Support for the third hypothesis would indicate that sperm length evolution is independent of sperm storage organ evolution. Thus, we also examined relations of sperm length with reproductive and life-history traits, and habitat specificity.

MATERIALS AND METHODS

Sampling and carrefour complexity

Data on the presence and complexity of sperm storage organs in the carrefour were gathered from 47 stylommatophoran snail and slug species representing 27 families. Nomenclature of gastropods follows Falkner et al. (2001a) (see Appendix 1 available online for a list of all species used). Species were chosen on the basis of three criteria: (1) the accessibility of living specimens or the availability of the required information on the morphology in the literature; (2) the representation of a wide range of stylommatophoran families in the set of species considered; and (3) the availability of 28S rDNA sequence data (for species where literature data were used).

Adult individuals were collected at various localities in Europe between June 2004 and March 2006. We removed the carrefour (C) from the proximal genital system, fixed it in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 16 h at room temperature and, subsequently, embedded it in paraplast or, for very small specimens, in Epon. After serially cross-sectioning and staining with haematoxylin-eosin or methylene blue azur II, respectively, the morphological structure of each carrefour was examined. We defined four complexity states: (C-1) carrefour forms a simple loop; no fertilization pouch and no spermatheca present; (C-2) fertilization pouch but no spermatheca present in carrefour; (C-3) beside fertilization pouch a spermatheca with a single tubule present, and (C-4) beside fertilization pouch either one spermathecal tubule with a highly structured wall or multiple spermathecal tubules present (both states potentially allowing for a spatial separation of allosperm stored). To minimize effects of intraspecific variation, we examined at least 3 individuals per species.

Reproductive characters

We considered traits which are assumed to be involved in postcopulatory selection processes (Davison et al., 2005; Koene & Schulenburg, 2005). Sperm storage may be

manipulated by the so-called love-dart shooting, in which calcareous darts laced with allohormones (Koene & ter Maat, 2001) from the dart-associated mucous glands are stabbed at the partner's skin during courtship (Chase & Blanchard, 2006). Information on the presence or absence of love darts was thus gathered during snail dissection or obtained from the literature. In several snail and slug species, other auxiliary copulatory organs beside love darts occur, often accompanied by glands or secretory cells, e.g., the ligula of Arionids or the digitiform penial gland of *Deroceras* (for an overview see Gómez 2001). These structures play an active role in mating and might possibly also influence sperm storage processes. Thus, we included a further binomial variable, which indicates the presence/absence of any kind of auxiliary copulatory organs (including love darts). Other structures within the reproductive tract that may influence the transfer of sperm to the storage organ are the flagellum (which produces the tail of the spermatophore) and the diverticulum (which is a part of the spermatophore receiving organs; Fig. 1). Flagellum length could be important because it has been hypothesized that sperm are most successful at reaching the storage organs, when the spermatophore's tail is protruding into the vaginal duct (Lind, 1973). On the other hand, the longer the diverticulum the longer the distance spermatozoa have to move to the sperm stores. For each species we categorized flagellum and diverticulum as present/absent, short (< than 20% of reproductive tract size) or long (≥ than 20% of reproductive tract size). Sample sizes differed among analyses because no data on reproductive traits were available for some species.

Data on the predominant mating system were extracted from the literature and from information provided by colleagues. The mating system of each species was classified as predominantly self-fertilization, mixed system or predominantly cross-fertilization. Because uncertainties and missing data occurred we analyzed the data of the mating system in two different ways: (1) using exclusively information available from the literature, and (2) using all available information from the species examined as well as information inferred from closely related species. Because both analyses revealed similar results, we chose to present the data including all available information.

Life history and habitat specificity

We collected data on the life-history traits adult size, longevity, reproductive strategy and reproductive mode from the literature. We scored adult size as small (maximum shell width, shell height or foot length [in slugs] < 12 mm) or large (≥ 12 mm). Longevity was scored as short (< 2 years) or long (≥ 2 years). The reproductive strategy was classified as

either semelparity (species that reproduce during one season, after which the animals die) or iteroparity (species that reproduce during two or more seasons). The reproductive mode was classified as oviparity (species that deposit eggs) or ovoviviparity (species that retain fertilized eggs in the female reproductive duct). We categorized habitat specificity following Falkner et al. (2001b) as: rock-dwelling species (species exclusively occurring on rocky substrates including cliff and scree; R), woodland (species mainly found in woodland; F), open-land (species exclusively occurring in open habitats; O) and ubiquitous species (species frequently found in more than one habitat type; U). All available data are presented in Appendix 1 and literature sources in Appendix 2 (available online). Samples sizes are, except for adult size, reduced because life-history traits of some species are unknown.

Carrefour length and sperm length

In species with a spermatheca in the carrefour, we measured the length of the whole complex on digital images obtained with a Sony CCD-Iris camera (Sony, Tokyo, Japan) mounted on a Leica MZ 8 binocular (Leica, Wetzlar, Germany). For sperm length measurements the central part of the hermaphroditic duct was put in a drop of Ringer-Solution (Romeis, 1989) on a microscopic slide. By carefully shaking the hermaphroditic duct, the spermatozoa were set free and subsequently air dried under a glass coverslip. Digital images of spermatozoa were obtained using a Sony CCD-Iris camera mounted on a Leica DML light microscope. For each snail we measured the total length (head and tail) of 25–30 randomly chosen sperm. All length measurements were determined using the public domain NIH Image program Version 1.63 (http://rsb.info.nih.gov/nih-image/). Carrefour length and sperm length were gathered from at least 3 specimens of each species and mean values were used in the analyses.

To correct for differences in body size among species, we calculated the residuals of carrefour length and sperm length by regressing (linear least-squares) the $\log_{10}$-transformed values of each continuous variable against $\log_{10}$-transformed shell width or height. Shell size, determined prior to dissection to the nearest 0.1 mm using a vernier calliper, is a more reliable measure of snail size than mass, because mass depends on the state of hydration and thus is highly variable in terrestrial gastropods. Because the body size of a slug is not comparable to the body size or shell size of a snail, we omitted slug data from the comparisons of carrefour length with sperm length, resulting in a sample size of 17 species.

To test for associations of sperm length with reproductive characters, life-history traits and habitat specificity, $\log_{10}$-transformed residual sperm length was assigned in ascending order to four length categories.

Molecular data and phylogenetic analyses

We used partial gene sequences near the 5'end of 28S rDNA for phylogenetic reconstructions. We determined sequences for 7 species and used published sequences of another 40 species (Wade et al. 2001, 2006; Armbruster et al. 2005; Koene and Schulenburg 2005; see Appendix 1 for GenBank Accession Nos.). In 12 cases, sequences from closely related species of the same genus were available. We used these data because genetic distances between species within a genus were minimal for 28S rDNA, ranging from 0 to 1.8% (Wade et al., 2001). This approach should not have any effect on the results.

DNA was extracted from a small amount of deep-frozen foot tissue (approximately 20 mg) following the DNeasy® Tissue Handbook of QIAGEN (2003) with proteinase K digestion over night and spin column treatment. Nucleic acids were eluted from spin column in a final volume of 150 μl AE buffer (QIAGEN, 2003). A 680 bp fragment near the 5'-end of the 28S rRNA gene was amplified with following primers: 28S-Forward: 5'-TCCGACCTCAGATCGGACGAGATTACC-3'; 28S-Reverse: 5'-GCGGTCGGGAGACACGGTTGCCCAGTC-3'. PCR was performed in 25 μl volume using PuReTaq™ Ready-To-Go™ PCR Beads (GE-Healthcare, 2006) with approximately 50 ng of DNA, 25 pmol of each primer and a final concentration of 1.5 mM of $MgCl_2$. PCR conditions were 95°C (1 min), primer annealing 50°C (30 sec) and polymerase extension 72°C (1 min), repeated in 35 PCR cycles. PCR products were checked for appropriate size in agarose gels. Amplified 28S rDNA fragments were purified using spin columns, and directly sequenced with an automated ABI sequencer (ECOGENICS GmbH, Schlieren; Switzerland). Both, the forward and reverse strands were sequenced. All sequences were deposited in GenBank (Accession Nos. EF010927 – EF010933).

Sequences of 47 stylommatophoran and three non-stylommatophoran pulmonates (Eupulmonata: *Melampus luteus*, Ellobiidae; *Carychium tridentatum*, Carychiidae; Basommatophora: *Siphonaria pectinata*, Siphonariidae) were aligned using the ClustalW online service (http://www.ebi.ac.uk/clustalw; Thompson et al. 1994) and optimized by eye. The aligned sequence region contained 567 positions. The phylogenetic relationships among the included taxa were addressed either using maximum likelihood (ML) as

implemented in the program PAUP* v.4.0b10 (Swofford, 2003) or Bayesian inference (BI) as implemented in the program MrBayes v.3.1.2. (Ronquist & Huelsenbeck, 2003). To find the most appropriate model of DNA substitution for our data, sequences were analyzed with Modeltest v. 3.7 (Posada & Crandall, 1998). Using the Akaike information criterion (Akaike, 1973), the resulting best fit model was GTR + Γ + I (general time-reversible model, with six rate classes, unequal base frequencies, a parameter for invariable sites and a gamma distributed rate heterogeneity parameter) with base frequencies of A = 0.1761, C = 0.2767, G = 0.3479, T = 0.2296; a rate matrix of [A–C] = 1.0990, [A–G] = 2.7639, [A–T] = 2.5802, [C–G] = 0.3575, [C–T] = 4.8149, [G–T] = 1.0000, a proportion of invariable sites of 0.5382, and a gamma distribution shape parameter of G = 0.7190.

For ML tree estimation, parameters of the substitution model (GTR + Γ + I) were first optimized using a neighbour-joining (NJ) tree as starting tree. The estimated parameters were then employed in the ML tree search, using the heuristic search options and branch-swapping by TBR. Because computation time was limited, the robustness of the tree was tested using nonparametric bootstrap analyses with 1000 replicates obtained from a NJ analysis of ML distances.

For the Bayesian analysis, the same model and parameters were applied using the MCMC procedure with two runs. For each run we used 1.000.000 generations, four chains (one cold and three heated) and a sampling frequency of 100 generations. From the 10.000 trees obtained, we determined a subset of trees for calculating a 50% majority rule consensus tree by inspecting likelihood values of trees saved by MRBAYES. The first 2.500 trees were discarded to ensure that stable likelihood values were achieved. The proportions of bifurcations found in the remaining 7.500 trees are given as posterior clade probabilities.

Ancestral character states and character association

Because of the potential confounding effects of shared ancestry in comparative studies, data collected from single species cannot necessarily be considered as independent observations (Felsenstein, 1985; Harvey & Pagel, 1991). However, the almost universally used parsimony method to map character states on a single phylogenetic tree is relying upon that this tree gives a valid representation of the hierarchical relationships among the species examined as well as their relative degrees of divergence. This method does also not account for the uncertainty in the process of character change. We therefore applied recently developed Bayesian methods that provide a framework explicitly accommodating

phylogenetic and character mapping uncertainties (Huelsenbeck et al., 2000; Ronquist, 2004).

We accommodated uncertainty in the phylogeny by averaging the ancestral character state reconstruction of carrefour complexity over the last 7.500 trees obtained from MrBayes using a hierarchical Bayesian approach (Nielsen, 2002) as implemented in SIMMAP v.1.0 Beta 2.3 (Bollback, 2006). To accommodate uncertainty in the overall rate of genital morphology evolution, a discrete γ prior was employed (Schultz & Churchill, 1999; Huelsenbeck et al., 2003). γ-distribution parameters, α and β, were chosen to be 1.0 and 0.2, respectively. The expected value and standard deviation for this distribution were 5.0 and 5.0, respectively. For each of the 7.500 trees, ten samples were drawn from the posterior distribution of the overall rate. We used the same γ prior to simulate the history of carrefour complexity evolution with SIMMAP. Testing different sets of the γ prior for both ancestral state reconstruction and character histories yielded similar results.

We repeated the ancestral character state reconstruction using both maximum likelihood and Bayesian inference with BayesMultistate v.1.0.2 (Pagel et al., 2004). The maximum likelihood approach incorporated in BayesMultiState uses a continuous time Markov model of character evolution. We used equal transition rates between carrefour complexity states because a model incorporating different rates did not lead to a significant improvement in the fit of the model compared to a model with a single rate (LR = 15.17, alpha = 0.05, critical value for χ^2 with 10 d.f. = 19.68; Schluter et al. 1997). Ancestral character states were estimated using the MRCA (most recent common ancestor) approach to work around the limitation of poorly supported nodes (Pagel et al., 2004). In the BI mode of BayesMultiState, ancestral character reconstruction analyses with the 7.500 trees were run for 75.000.000 generations, with sampling of parameter values every 5.000 generations and a flat prior distribution (ranging from 0 to 100). The first 10% of the sample was discarded as burn-in, with the remaining 90% of the sample used for estimation of the posterior probabilities for the ancestral character states of the most recent common ancestors of various clades.

Associations of carrefour complexity and sperm length categories with reproductive characters, life-history traits and habitat specificity were calculated with SIMMAP using a subset of trees (because each analysis took very long, i.e., > 80 hours), with the last 900 trees sampled every 1.000th generation in a Bayesian analysis and employing flat, uninformative priors (γ-rate parameter α = 1.0, β = 0.2, k = 50; β = 1, k =

5). For each of the 900 trees, five samples were drawn from the posterior distribution of the overall rate and bias. Based on character histories, state-by-state character associations (*d*) and overall character association (*D*) were calculated by examining the difference between the observed and expected values for each combination of states (Huelsenbeck et al., 2003). The value of *d* is negative if two specific states are found together less frequently than would be expected under independence, and positive if they are found together more frequently than expected. The statistic *d* depends upon the tree, branch lengths and character mappings for the two characters. The overall measure *D* represents the disagreement between the observed and expected associations of the states for the two characters. The posterior predictive *P* value is calculated by simulating a large number of character histories under the assumption that the two characters are independent (Huelsenbeck et al., 2003). Observed values were considered significantly different from expected if they fell outside 95% of the probability density of the simulated distribution. The results were largely robust when different prior sets or an increased number of samples drawn from the priors were used.

To test for correlated evolutionary changes between carrefour length and sperm length, we either used bivariate analyses of species data or data after phylogenetic correction with a generalized least-squares (GLS) approach as implemented in the program Continuous (Pagel, 1997; Pagel, 1999). The program uses maximum likelihood models to investigate correlations between continuously varying characters while controlling for phylogenetic associations by reference to an internal matrix of expected covariances among species owing to their degree of shared ancestry, without the need to calculate independent contrasts (Pagel, 1999). It allows to incorporate three scaling parameters into the data analysis, which significantly improves the fit of the data to the model: (1) κ characterizes the mode of evolution, (2) δ detects different rates of evolution over time, and (3) λ reveals whether the phylogeny correctly predicts the patterns of covariance among species on a given trait. Continuous estimates these parameters by maximum likelihood. Zero branch lengths were set to 10^{-4}, because Continuous cannot accept branch lengths smaller than or equal to zero.

RESULTS

Carrefour complexity and spermatheca evolution

The distribution of different carrefour morphologies as well as the Bayesian reconstruction of ancestral states are shown in the 50% majority-rule consensus tree from the Bayesian phylogeny (Figure 2). The analyses of the last 7.500 trees of MrBayes using SIMMAP provide evidence that, with a posterior probability of 0.70, the common ancestor of stylommatophoran snails and slugs possessed a carrefour with a fertilization pouch but without a sperm storage organ (C-2; see pie charts in Fig. 2). The results obtained with BayesMultistate, using both maximum likelihood and Bayesian methods, were largely similar to those obtained with SIMMAP in the well-supported nodes, but differed in nodes with low bootstrap values and posterior probabilities (e.g., the two nodes marked with an asterisk in Fig. 2).

The pattern of carrefour complexity evolution simulated with SIMMAP suggests more than ten character transformations. The gain of a spermatheca with a single (C-3) or multiple sperm storing tubules (C-4) occurred independently at least two times and from all possible states three times. Beside the large group of Helicoidea/Discidae with complex sperm storage organs within their carrefour, *Succinea* has a spermatheca with two sperm storing tubules (Rigby, 1965) and the Orthalicidae, represented by *Bulimulus* and *Drymaeus*, have spermathecae with a high number of tubules (van Mol, 1971). Moreover, in the group of Limacoidea several species occur that possess a spermatheca with one sperm storing tubule (e.g., *Deroceras*, *Vitrina* and *Oxychilus*).

Our analyses also indicate that a spermatheca was lost independently from the carrefour more than two times, in some species together with the fertilization pouch (in *Arion* and *Philomycus*). The transition of a spermatheca with multiple sperm storing tubules to a spermatheca with a single tubule occurred two times independently in the group of Helicoidea (in *Fruticola* and *Xerosecta*).

Phylogeny

Repeated Bayesian phylogenetic analyses of the partial 28S rDNA dataset revealed similar posterior probabilities, indicating insensitivity to the starting tree. Tree topologies were also concordant with maximum likelihood phylogeny reconstruction. Moreover, except for a few nodes, the clades with high posterior probabilities also had strong bootstrap support.

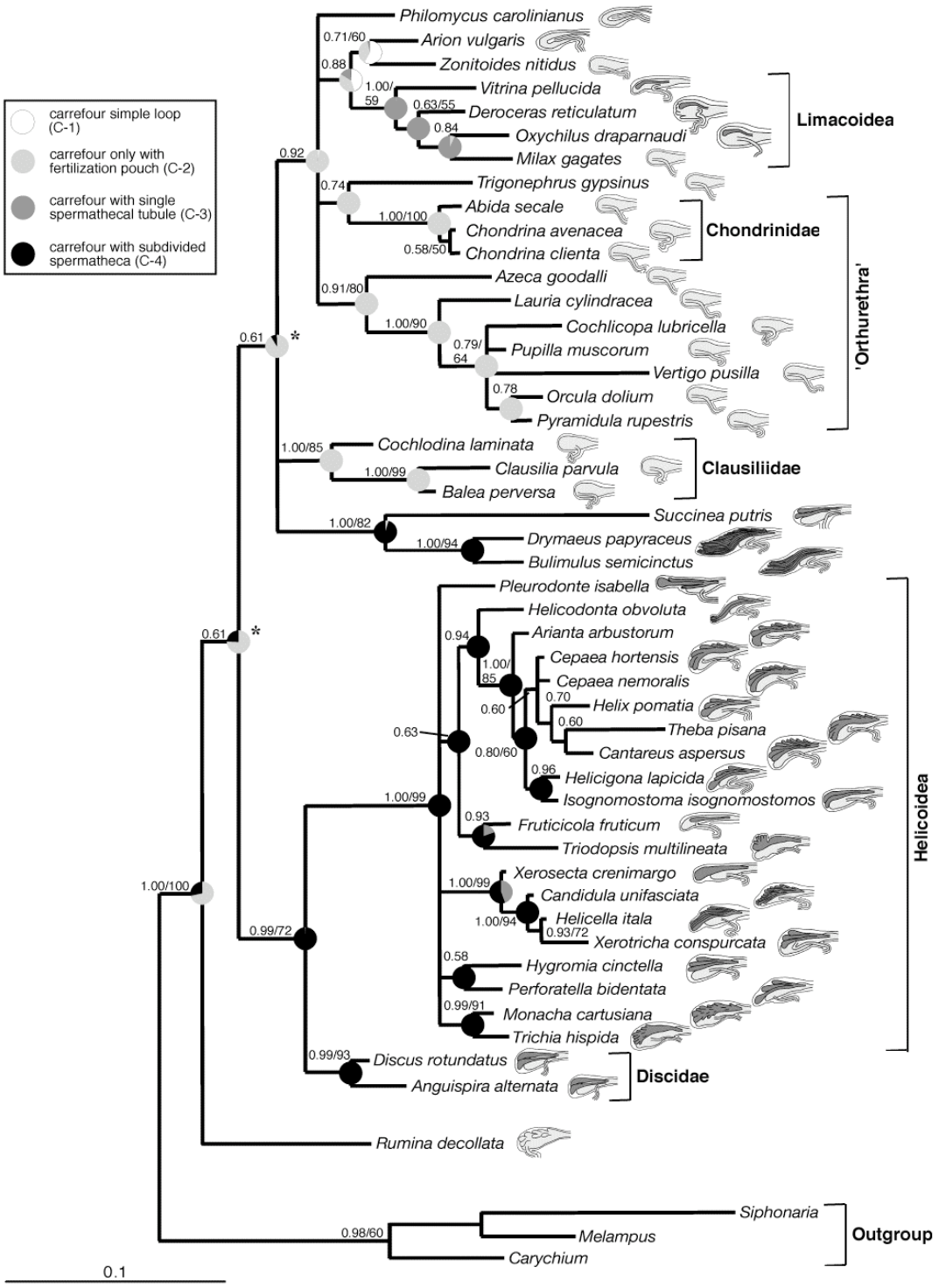

Figure 2. Phylogenetic relationships of stylommatophoran species with reconstruction of ancestral character states of carrefour complexity. Tree topology is based on partial 28S rDNA alignments. The branch lengths of the 50% majority rule consensus tree of the Bayesian analysis correspond to the number of substitutions per site. Numbers on the branches are Bayesian posterior probabilities larger than 0.5 and bootstrap values larger than 50 separated by a slash. The schematic drawings of carrefour region are scaled to the same size for graphical presentation. The fertilization pouch of the carrefour is depicted in light gray, the spermatheca in dark gray. Pie charts on the nodes indicate the posterior probabilities for Bayesian estimates of ancestral character states of carrefour complexity. Asterisks are explained in the text.

As in previously published stylommatophoran phylogenetic analyses (Wade et al., 2001; 2006), most groups, e.g., Helicoidea and Limacoidea, have been correctly identified, but the phylogenetic resolution remained low at the base. The superfamily of Helicoidea (including Bradybaenidae, Camaenidae, Helicidae, Helicodontidae, Hygromiidae and Polygyridae) and the families of Chondrinidae, Clausiliidae and Discidae were well supported with posterior probabilities larger than 0.99 and bootstrap values above 85%. The previously proposed monophyly of both the Orthurethra and the Arionoidea (Wade et al., 2006) was rejected in our phylogenetic reconstruction.

Carrefour associations with reproductive characters

The results of the character association analyses of carrefour complexity with reproductive characters are summarized in Table 1. The overall character association (*D*) was high for carrefour complexity with dart shooting ($D = 0.549$) and flagellum ($D = 0.575$), and the predominant mating system ($D = 0.713$). However, *D* accounts for associations in all pairwise comparisons of states and may mask strong relationships between individual characters. The comparison of state-by-state associations (*d*) revealed a number of significantly positive as well as negative associations of carrefour complexity state with reproductive characters. Specifically, significant positive associations were found between the occurrence of dart shooting or the presence of any kind of auxiliary copulatory organ and a carrefour with a subdivided spermatheca (C-4). Similarly, the absence of dart shooting and an auxiliary copulatory organ was positively associated with a carrefour consisting only of a fertilization pouch (C-2). Furthermore, the presence of a long flagellum was positively associated with C-4, and the absence of both a flagellum and a diverticulum with C-2. Finally, we found a significant positive association between predominant cross-fertilization and C-4, while both predominant self-fertilization and mixed mating system were associated with C-2.

Associations with life history and habitat specificity

Various life-history characters and habitat specificity were associated with carrefour complexity (Table 1). Small adult size and ovoviviparity were positively associated with C-2, whereas iteroparity and oviparity were positively associated with C-4. Large adult size and oviparity were associated with C-2, as was semelparity with C-4. Regarding habitat specificity, species occurring on rocks or cliffs most likely possessed C-2, while woodland species predominantly had C-4.

Table 1. Character associations of carrefour complexity with reproductive characters, life-history traits and habitat specificity estimated using SIMMAP. The results are based on the last 900 trees sampled every 1.000th generation in a Bayesian analysis. State-by-state character associations (*d*) are positive if two specific states are found together more frequently than expected under independence and negative if two states are found together less frequently than expected. The significance of *d* is given as: ** $P < 0.01$, and * $P < 0.05$.

			Carrefour complexity				
			State-by-state character associations (*d*)				Overall character association (*D*)
	Trait	Character state	C-1	C-2	C-3	C-4	
Reproductive characters	Dart shooting	absent	0.004	**0.133****	0.017	**-0.120****	0.549
		present	-0.002	**-0.112****	-0.012	**0.149***	
	Auxiliary copulatory organ (including dart)	absent	-0.011	**0.115***	-0.012	**-0.069***	0.428
		present	0.013	**-0.093***	0.018	**0.097***	
	Flagellum	absent	0.015	**0.110****	0.018	**-0.143****	0.575
		short	-0.006	-0.032	0.002	0.036	
		long	-0.008	**-0.078***	-0.020	**0.106****	
	Diverticulum	absent	0.006	**0.054***	0.015	-0.026	0.204
		short	0.000	-0.001	-0.001	0.003	
		long	-0.004	**-0.033***	-0.009	0.052	
	Predominant mating system	self-fertilization	-0.006	**0.123***	-0.020	**-0.082***	0.713
		mixed	0.022	0.029	0.032	**-0.068***	
		cross-fertilization	-0.015	**-0.131***	-0.007	**0.178****	
Life history and habitat specificity	Size	small	-0.007	**0.098***	-0.023	-0.042	0.355
		large	0.009	**-0.077***	0.028	0.071	
	Longevity	< 2 years	0.014	-0.023	0.038	-0.003	0.199
		≥ 2 years	-0.012	0.044	-0.032	0.032	
	Reproductive strategy	semelparity	0.012	0.033	0.027	**-0.037***	0.217
		iteroparity	-0.010	-0.011	-0.022	**0.065***	
	Reproductive mode	oviparity	0.007	**-0.083***	0.021	**0.101***	0.408
		ovoviviparity	-0.005	**0.104***	-0.016	**-0.072***	
	Habitat	rock/cliff/scree	-0.003	**0.082***	-0.006	**-0.064***	0.404
		woodland	-0.003	-0.015	-0.026	**0.065***	
		open-land	-0.002	-0.034	0.045	0.011	
		ubiquitous	0.010	-0.012	-0.008	0.017	

Variation in carrefour length and sperm length

Carrefour length and sperm length varied substantially across stylommatophoran species (Appendix 1). The smallest carrefour with a spermatheca was found in *Xerotricha conspurcata* (mean length of 0.4 mm), the largest in *Arianta arbustorum* (3.1 mm). Mean sperm length ranged from 134 μm in *Deroceras reticulatum* to 1170 μm in *Cochlodina laminata*. Both traits were positively related to maximum shell width (carrefour length: $r^2 = 0.80$, $F_{1,15} = 60.67$, $P < 0.001$; sperm length: $r^2 = 0.35$, $F_{1,15} = 8.14$, $P = 0.012$).

Considering species with a spermatheca in their carrefour, we found a significant correlation between residual sperm length and residual carrefour length ($r = 0.50$, $n = 17$, $P = 0.041$; see Fig. 3). This was consistent with the result when phylogeny is taken into account, using a model that incorporated the ML estimates of the three scaling parameters κ, δ and λ ($n = 17$, $R = 0.465$, $P = 0.042$; $\kappa = 0.93$, $\delta = 2.97$, $\lambda = 0.81$). A δ larger than 1 suggests that species-specific adaptation has been dominant over adaptive radiation. However, using likelihood ratio tests (LRT), both κ and δ were not significantly different from one. Nevertheless, κ was significantly different from zero ($P = 0.006$) and therefore trait evolution is consistent with some form of gradual mode of trait evolution. Moreover, the model incorporating the ML estimate of λ was marginally significantly preferred over a model in which λ was constrained to one ($P = 0.068$). This suggests that the data did not fit the Brownian model of evolution. The preferred model was also not significantly different from another model constraining λ to zero (species values are independent).

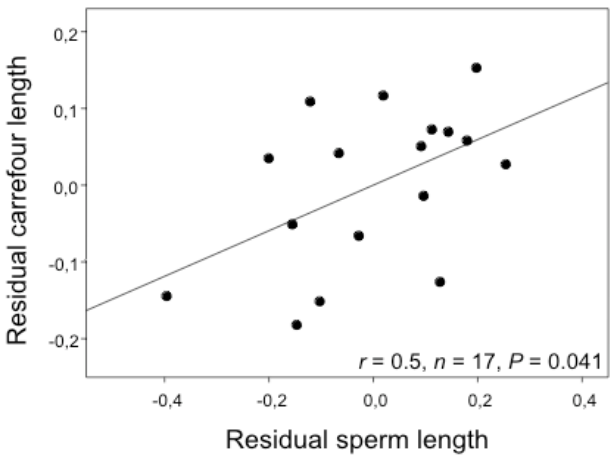

Figure 3. Relationship between residual carrefour length and residual sperm length. Carrefour length and sperm length were controlled for maximum shell width. Data were sampled across all stylommatophoran snail species that possess a spermatheca within their carrefour and are based on bivariate analyses of species data. The GLS approach using Continuous (Pagel, 1997; Pagel, 1999) gave virtually equal results.

There were no associations of the four sperm length categories with either reproductive characters, life-history traits or habitat specificity (results not shown), except for a negative association of long sperm (category 3) with semelparity ($P < 0.05$).

DISCUSSION

Our phylogenetic analyses indicate that in stylommatophoran gastropods female sperm storage organs have originated in the carrefour more than once, and that they were secondarily lost several times. Even the fertilization pouch was lost in some lineages. Our study also revealed an extensive evolutionary diversification of sperm storage organs in several groups of hermaphroditic snails and slugs, similar to that in gonochoric animals (Eberhard 1985; Pitnick et al. 1999). These results suggest that in hermaphroditic animals equally strong selective forces are driving the evolution of female reproductive morphology than in species with separated sexes.

Stylommatophoran gastropods have undergone an explosive radiation during the Late Cretaceous or Early Tertiary (Tillier et al., 1996), resulting in a low support of basal nodes as found in the present study, and in earlier work based on ribosomal RNA gene trees (Wade et al., 2006) and histone gene trees (Armbruster et al., 2005). Despite of shorter ribosomal DNA sequences, our phylogenetic tree is similar to that of Wade et al. (2006). We tried to minimize uncertainties limiting our conclusions about sperm storage organ evolution by using different methods of phylogeny reconstruction and character history estimation. Bayesian and maximum likelihood methods revealed similar results. Our study also showed that a phylogenetic correction is not necessarily required when comparing sperm length and carrefour length evolution in stylommatophoran gastropods.

The majority of 'orthurethran' snails, the Clausiliidae and a few other taxa (e.g., *Milax*, *Zonitoides*) possess no spermatheca in their carrefour. This condition seems to be ancestral in stylommatophoran species, and is consistent with the observation that basommatophoran snails, one outgroup of stylommatophoran pulmonates, have no sperm storage organ in their carrefour (Abdel-Malek, 1954; Tomé & Ribeiro, 1998). Nevertheless, some basommatophoran species preferentially use allosperm for fertilization even several months following mating, and thus a spatial separation of auto- and allosperm seems to be possible (Jarne et al., 1993). It remains, however, unclear how and where allosperm are stored in basommatophorans, and if stylommatophoran species without a spermatheca also can separate allo- from autosperm. Unfortunately, parentage analyses of

stylommatophorans with a simple carrefour are lacking. The highly complex spermathecae with multiple sperm storing tubules, which occur in Helicoidea, Discidae, *Succinea*, *Bulimulus*, and *Drymaeus*, suggest that selection has favored their evolution in such a way that the benefits to the female function outweigh the costs of developing, maintaining and using these structures.

Reproductive characters and postcopulatory sexual selection

In the group of Helicoidea, support for repeated and correlated evolution of the two male traits dart shape and mucus gland complexity with spermatophore-receiving organs belonging to the female part have been found, and were interpreted as evidence for sexually antagonistic counteradaptations resulting from sexual conflicts (Koene & Schulenburg, 2005). Similarly, our results suggest that the evolution of spermathecae is associated with the presence of dart shooting, but also with the presence of any kind of auxiliary copulatory organ, e.g., the stimulator of Vitrinidae (although secondarily lost in the genus *Vitrina*; Hausdorf 1998) or the digitiform penial gland in the genus *Deroceras*. Because a female-mediated control of paternity is only possible when a spermatheca is present, it can be hypothesized that the co-occurrence of sperm storage organs and auxiliary copulatory organs, including love-darts, also is a result of antagonistic coevolution. However, in discussing this association one has to consider that, apart from inevitable uncertainties concerning the order of trait evolution, our knowledge on the manipulation of sperm storage processes by dart-shooting is still limited except in *Cornu aspersum*, a helicid snail (Chase & Blanchard, 2006). Furthermore, information on the distribution and function of other auxiliary copulatory organs is poor, and the homology of these structures is unknown. We also found several exceptions for the co-occurrence of auxiliary copulatory organs and spermathecae. For example, *Succinea* possesses a complex spermatheca but no dart or any penial appendages, and although *Philomycus*, *Arion*, *Zonitoides*, and *Milax* possess auxiliary copulatory organs, no spermatheca is found in the carrefour of these species. These results require further investigations.

Combining the findings of Koene and Schulenburg (2005) with our results suggests that a set of male and female reproductive characters is involved in the antagonistic selection. The presence of flagellum (male part) and diverticulum (female part) may be adaptive when these structures increase the own reproductive success or allow control over allosperm storage. Indeed, the presence of a long flagellum was linked with spermatheca presence, and the absence of flagellum and diverticulum with spermatheca absence.

However, because a long flagellum predominantly is found in Helicoidea, which also possess love darts, traits manipulating sperm storage could mainly include dart shooting and other traits that are only indirectly related to spermatheca evolution.

Obligatory self-fertilization should exclude any possibility of postcopulatory sexual selection. In fact, specialized sperm storage organs were absent from the carrefour of predominantly selfing species. Furthermore, no spermatheca was found in most species with a mixed mating system. In contrast, all species with predominant cross-fertilization had at least one spermathecal tubule. In most outcrossers either one spermathecal tubule with a highly structured wall or multiple tubules were present. This may allow for a spatial separation of stored sperm, a prerequisite for cryptic female choice (Hellriegel & Ward, 1998). These findings are consistent with the hypothesis that carrefour divergence is due to differences in the mating system.

There are, however, other explanations possible for the interpretation of our results. Carrefour morphology may be an evolutionary conservative trait and the relationship between mating system and carrefour complexity could be the result of a strong correlation with phylogeny. This could be tested by examining carrefour differentiation of closely related species that differ in their mating system. However, mating systems also vary within species because of differences in population density and local environmental conditions. To minimize errors due to misclassified species, we used the mating system categories predominantly selfing, outcrossing and mixed mating. To the latter category we assigned all species where the available information was ambiguous, i.e., several authors reported on frequent outcrossing but records on selfing were also found. Moreover, our results considering all available information on the mating system, including inferences from closely related species, did not differ from the analyses were we only included information on the specific species. Thus, we believe our assignments to the mating system were largely consistent with the situation in nature.

The numbers of species with a carrefour consisting of only a simple loop and that of species with a spermatheca consisting of a single tubule, however, were very low and this could have influenced our results of reproductive character associations. In addition, data on mate availability, mate-choice, and mating rates should also be gathered for future investigations on the evolution of female sperm storage organs.

Influence of life history and habitat specificity

Life-history evolution may affect interspecific variation in the expression of sexually selected traits (Badyaev, 1997). An association between small body size and internal brooding has been found in a variety of marine invertebrate taxa (e.g., echinoderms, bivalves; Strathmann 1985, 1990). Due to allometric constraints small individuals achieve a higher reproductive success by brooding, whereas in large individuals the production of a large number of eggs is the best reproductive strategy (Strathmann & Strathmann, 1982; Baur & Raboud, 1988; Baur, 1994b). In contrast to larger species that lay many eggs, small species might not require a specialized organ for long-term sperm storage because they produce only a few offspring in their lifetime. The reproductive mode may thus have influenced the evolution of sperm storage organs, resulting in the association of small body size and ovoviviparity with spermatheca absence, and oviparity with spermatheca presence. Alternatively, small body size and ovoviviparity could be adaptations to habitats with harsh environmental conditions that provide no suitable oviposition sites, e.g., rock walls (Baur, 1994b; Heller, 2001). The exposed habitats may furthermore have favored self-fertilization, which secures reproductive success when the probability of finding mates is restricted. It is assumed that selfing species do not require storage organs for allosperm. Thus, spermatheca absence could also be a result of the intertwined forces of life history, mating system, and habitat in which the animals live. Because the majority of small and ovoviviparous species belong to few gastropod families, a phylogenetic effect on the observed distribution of spermatheca presence cannot be excluded.

Viable allosperm can be stored for more than one year in species with long life span, as in Achatinidae and Helicidae (Lind, 1973; Baur, 1998). Nevertheless, in contrary to our expectation, we found no association between spermatheca presence and longevity. Longevity seems thus not to reflect the reproductive strategy in stylommatophorans. Indeed, several of the long-living species examined in our study were semelparous, which was negatively related to spermatheca presence. In iteroparous species, however, a complex spermatheca was found supporting the hypothesis that specialized organs are indicative of long-term sperm storage.

Carrefour size and sperm length evolution

Sperm size is a highly variable trait both inter- and intraspecific (Ward, 1998b; Snook, 2005), also in stylommatophoran gastropods (Thompson, 1973; Minoretti & Baur, 2006).

In our analysis, residual carrefour length in the stylommatophoran gastropods with a spermatheca proved to be positively correlated with residual sperm length, but sperm length did not exhibit any association with other reproductive characters, life-history traits or habitats specificity. These findings support the suggestion that sperm length divergence results from sperm size evolving in response to changing female morphology (e.g., Pattarini et al. 2006). Experimental evolution studies showed that changing female spermatheca length can drive the divergence in sperm length (Miller & Pitnick, 2002; Miller & Pitnick, 2003). In addition, Pitnick et al. (1999) noted that sperm length changes across different species of *Drosophila* were rather slow compared with spermatheca size changes. Thus, the physical characteristics of female sperm storage organs may impose stabilizing selection on sperm length (Simmons & Kotiaho, 2002). Moreover, longer sperm of *Drosophila* have a fertilization advantage by occupying and/or retaining occupancy in the storage organ better, or being better in displacing or resisting being displaced by shorter sperm (Pattarini et al., 2006).

In helicid land snails allosperm 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 (Bojat et al., 2001b; Rogers & Chase, 2002). 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 (Rogers & Chase, 2002); with a larger sperm size and a higher sperm number resulting in a stronger resistive force (Beese et al., 2006). Blocking of the storage organ for sperm from future mates, in turn, may have favored divergence in spermatheca length. Longer storage organs could allow the female function to take up more sperm and thereby to benefit from a greater control over the fertilization process (Eberhard, 1996; Pitnick et al., 1999; Miller & Pitnick, 2003). This could have resulted in a coevolution leading to the association between sperm length and female sperm storage organ length found across snail species in the present work but also across other animal taxa (Dybas & Dybas, 1981; Briskie & Montgomerie, 1992; Pitnick et al., 1999; Presgraves et al., 1999; Morrow & Gage, 2000).

We further expected to find a relationship between sperm length and the predominant mating system. Because the production of long sperm is costly (Pitnick et al., 1995; Pitnick, 1996), the relaxed selection pressure on males should have led to the evolution of short or aflagellate sperm as reported for other taxa (LaMunyon & Ward, 1998; Morrow, 2004). However, our data did not confirm any reduction of sperm length in species that predominantly self-fertilize.

Conclusions

Recent studies indicate that sexual selection and sexual conflict frequently signify high mating costs, and that mate harm may reach higher levels in hermaphrodites than in gonochorists (Michiels & Koene, 2006). Postcopulatory sexual selection may also have influenced the origin and diversity of female sperm storage organs in stylommatophoran gastropods, consistent with the recent hypothesis of inter-sexual counteradaptations driving correlated reproductive character evolution in stylommatophoran gastropods (Davison et al., 2005; Koene & Schulenburg, 2005; Beese et al., 2006). Moreover, our results revealed that life-history traits and habitat specificity have potentially influenced the evolution of female reproductive morphology and should be taken into account in future studies of reproductive trait divergence. With the increasing number of comparative studies, we now begin to understand the evolution of the highly diverse and complex reproductive characters in stylommatophoran gastropods.

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APPENDIX

Appendix 1 – Taxonomic information, sampling locality, accession numbers, carrefour complexity categories, reproductive characters, life-history traits, habitat specificity, carrefour length, and sperm length of the species used in our analyses. a-absent, p-present; l-large, s-small; R-rock-dwelling (including cliff and scree), F-woodland, O-open-land, and U-ubiquitous species.

Family	Species	Sampling locality	Genbank accession nr	Carrefour complexity	Dart	Auxiliary copulatory organ (incl. dart)	Flagellum	Diverticulum	Predominant mating system	Size	Longevity	Reproductive strategy	Reproductive mode	Habitat specificity	Carrefour length (mm)	Sperm length (µm) (category)
Cochlicopidae	*Cochlicopa lubricella* (Rossmässler, 1834)	Skogsby; Sweden	AY0144020	2	a	?	?	?	selfing	s	≥ 2 yrs	?	?	O	–	328 (3)
Ateeidae	*Azeca goodalli* (Férussac, 1821)	Alicas; Spain[1]	AY546470	2	a	a	?	?	?	s	?	?	?	F	–	?
Pupillidae	*Pupilla muscorum* (L., 1758)	Ekelunda; Sweden	EF10933	2	a	?	a	?	mixed	s	?	?	ovoviviparity	O	–	187 (2)
Lauriidae	*Lauria cylindracea* (da Costa, 1778)	Bretagne; France	AY0144023	2	a	?	short	a	selfing[*]	s	≥ 2 yrs	?	ovoviviparity	U	–	468 (4)
Valvatidae	*Valvata pusilla* Müller, 1774	Skogsby; Sweden	AY0144027	2	a	?	a	a	mixed	s	< 2 yrs	?	oviparity	F	–	203 (2)
Orculidae	*Orcula dolium* (Draparnaud, 1801)	Blauen; Switzerland	AY0144028	2	a	?	a	a	?	s	?	?	?	R	–	429 (3)
Pyramidulidae	*Pyramidula pusilla* (Vallot, 1801)	Gsellingen; Switzerland	AY0144030	2	a	?	?	?	selfing	s	?	?	ovoviviparity	R	–	336 (3)
Chondrinidae	*Abida secale* (Draparnaud, 1801)	Gsellingen; Switzerland	EF10929	2	a	a	a	a	mixed[#]	s	≥ 2 yrs	?	?	R	–	386 (2)
	Chondrina avenacea (Bruguière, 1792)	Gsellingen; Switzerland	AY0144032	2	a	a	a	a	selfing	s	?	?	?	R	–	379 (2)
	Chondrina clienta (Westerlund, 1883)	Torslunda; Sweden	AY0144031	2	a	a	a	a	selfing	s	≥ 2 yrs	?	oviparity	?	–	330 (2)
Clausiliidae	*Cochlodina laminata* (Montagu, 1803)	Aesch; Switzerland	AY0144047	2	a	a	a	a	?	l	≥ 2 yrs	?	?	F	–	1170 (4)
	Clausilia augosa Férussac, 1807	Gsellingen; Switzerland	AY0144051	2	a	a	a	a	mixed[#]	s	?	?	?	F	–	728 (4)
	Balea perversa (L., 1758)	Torslunda; Sweden	EF10932	2	a	a	a	a	selfing	s	?	?	ovoviviparity	R	–	749 (4)
Succineidae	*Succinea putris* (L., 1758)	Shinfield; Great Britain[2]	AY0144057	4	a	a	a	a	outcrossing	l	≥ 2 yrs	?	?	R	–	385 (2)
Orthalicidae	*Bulimulus sem'nitidus* Pilsbry, 1897	Guadeloupe[a]	AY841299	4	a	?	short	a	?	l	< 2 yrs	?	?	?	–	?
	Drymaeus papyraceus (Mawe, 1823)	Rio de Janeiro; Brasil[a]	AY841300	4	a	?	short	?	?	l	?	?	?	F	–	?
Subulinidae	*Rumina decollata* (L., 1758)	Lanzarote; Spain	AY0144065	2	a	?	short	a	selfing	l	< 2 yrs	semelparity	oviparity	O	–	553 (2)
Dorcasiidae	*Trigonephrus gypsinus* (Melvill & Ponsonby, 1891)	Vaalputs; South Africa[a]	AY0144081	2	a	?	short	a	?	l	?	?	?	?	–	?
Discidae	*Anguispira alternata* (Say, 1816)	[a]	AY841309	4	a	a	?	?	?	l	?	?	oviparity	F	–	557 (2)
	Discus rotundatus (Müller, 1774)	Gempen; Switzerland	AY0144097	4	a	a	a	a	?	s	< 2 yrs	?	?	F	–	439 (3)
Vitrinidae	*Vitrina pellucida* (Müller, 1774)	Skogsby; Sweden	AY0144111	3	a	a	a	a	mixed	s	< 2 yrs	semelparity	oviparity	O	0.7	203 (1)
Oxychilidae	*Oxychilus draparnaudi* (Beck, 1837)	Basel; Switzerland	AY0144115	3	a	a	short	a	mixed[#]	l	< 2 yrs	semelparity	oviparity	R	1.1	191 (1)
Milacidae	*Milax gagates* (Draparnaud, 1801)	Stellenbosch; South Africa[a]	AY0144117	2	a	p	a	a	mixed	l	< 2 yrs	semelparity	oviparity	O	–	?
Agriolimacidae	*Deroceras reticulatum* (Müller, 1774)	Sassnitz; Germany	AY0144119	3	a	p	a	a	outcrossing	l	< 2 yrs	semelparity	oviparity	O	0.7	134
Polygyridae	*Triodopsis multilineata* (Say, 1821)	'	AY841316	4	a	?	?	?	outcrossing[a]	l	?	?	?	?	–	?
Camaenidae	*Pleurodonte isabella* (Férussac, 1821)	'	AY841322	4	a	a	short	a	?	l	?	?	?	?	–	?
Hygromiidae	*Monacha cartusiana* (Müller, 1774)	Birsfelden; Switzerland	AJ560962	4	a	a	long	a	mixed[#]	s	< 2 yrs	semelparity	oviparity	O	0.9	339 (2)
	Trichia hispida (L., 1758)	Moscow; Russia[1]	AY0144125	4	p	p	long	a	outcrossing[a]	s	≥ 2 yrs	semelparity	oviparity	O	1.1	300 (2)
	Helicella itala (L., 1758)	Bretagne; France	AJ560963	4	p	p	short	a	outcrossing[a]	l	≥ 2 yrs	semelparity	oviparity	U	1.1	358 (1)
	Candidula unifasciata (Poiret, 1801)	Sassnitz; Germany	EF10930	4	p	p	short	a	outcrossing[a]	s	< 2 yrs	?	oviparity	O	0.9	304 (2)
	Hygromia cinctella (Draparnaud, 1801)	Basel; Switzerland	AJ560968	4	p	p	short	a	mixed[#]	s	?	?	oviparity	U	1.2	380 (2)
	Perforatella bidentata (Gmelin, 1791)	'	AJ560966	4	p	p	?	?	outcrossing[a]	s	< 2 yrs	semelparity	oviparity	F	–	?
	Xerocrassa cremnicarpa (Pfeiffer, 1848)	'	AJ560958	3	p	p	?	?	outcrossing[a]	l	?	?	oviparity	O	–	?
	Xerotricha conspurcata (Draparnaud, 1801)	St. Vio; France	AJ560964	4	p	p	long	a	outcrossing[a]	s	?	?	?	?	0.4	371 (3)
Bradybaenidae	*Fruticicola fruticum* (Müller, 1774)	Seewen; Switzerland	AJ560971	3	p	p	a	a	outcrossing[a]	l	≥ 2 yrs	?	?	O	2.3	360 (1)
Helicodontidae	*Helicodonta obvoluta* (Müller, 1774)	Nenzlingen; Switzerland	EF10931	4	a	p	a	a	outcrossing[a]	l	?	?	oviparity	U	1.4	635 (3)
Helicidae	*Arianta arbustorum* (L., 1758)	Nuglar; Switzerland	AY0144136	4	p	p	long	long	outcrossing	l	≥ 2 yrs	?	oviparity	F	3.1	913 (3)
	Helicigona lapicida (L., 1758)	Mokleby; Sweden	AY0144137	4	p	p	long	long	outcrossing[a]	l	?	?	oviparity	U	1.8	633 (3)
	Isognomostoma isognomostomos (Schröter, 1784)	Pfeffingen; Switzerland	EF10928	4	p	p	long	long	outcrossing[a]	s	?	?	oviparity	F	0.9	646 (3)
	Cepaea hortensis (Müller, 1774)	Skogsby; Sweden	AY0144131	4	p	p	long	a	outcrossing	l	≥ 2 yrs	?	oviparity	F	2.4	784 (3)
	Cepaea nemoralis (L., 1758)	Prora; Germany	AY0144130	4	p	p	long	a	outcrossing	l	≥ 2 yrs	?	oviparity	O	2.6	735 (3)
	Theba pisana (Müller, 1774)	Bretagne; France	AY0144135	4	p	p	a	long	outcrossing	l	< 2 yrs	semelparity	oviparity	O	2.5	729 (3)
	Cornu aspersum (Müller, 1774)	Bretagne; France	AY0144128	4	p	p	long	long	outcrossing	l	≥ 2 yrs	?	oviparity	F	2.9	697 (2)
	Helix pomatia L., 1758	Copenhagen; Denmark[3]	AJ560974	4	p	p	long	short	outcrossing[a]	l	≥ 2 yrs	?	oviparity	U	–	850 (2)
Gastrodontidae	*Zonitoides nitidus* (Müller, 1774)	Sassnitz; Germany	EF10927	2	p	p	a	a	selfing	s	< 2 yrs	semelparity	oviparity	F	–	185 (1)
Arionidae	*Arion vulgaris* Moquin-Tandon, 1855	Münchenstein; Switzerland	AY0144144	1	a	p	a	a	mixed	l	< 2 yrs	semelparity	oviparity	U	–	343
Philomycidae	*Philomycus carolinianus* (Bosc, 1802)	Urbana, IL; USA[a]	AY841340	1	p	p	a	a	mixed	l	?	?	?	?	–	?

Data on carrefour complexity were obtained in this study or from the literature: [1]Gómez and Angulo 1990, [2]Rigby 1965, [3]van Mol 1971, [4]Brinders and Sirgel 1992, [5]Gugler 1964, [6]Els 1974, [7]Schileyko and Schileyko 1975, [8]Lind 1973, [9]Kugler 1965.
*Data on the mating system were inferred from closely related species.

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CHAPTER II

COEVOLUTION OF MALE AND FEMALE REPRODUCTIVE TRAITS IN A SIMULTANEOUSLY HERMAPHRODITIC LAND SNAIL

Kathleen Beese, Konstantin Beier & Bruno Baur

ABSTRACT

Inter- and intraspecific studies in gonochoristic animals reveal a covariation between sperm characteristics and the size of the female reproductive tract, indicating a rapid evolutionary divergence, which is consistent with the theory of postcopulatory sexual selection. Simultaneous hermaphrodites differ from species with separate sexes (gonochorists) in that they possess both functional male and female reproductive organs at the same time. We investigated whether in hermaphroditic animals intraspecific variation in reproductive traits results from divergent coevolution, by quantifying the variation in male and female traits among six natural populations of the snail *Arianta arbustorum* and examining the covariation in interacting traits. There was a significant among-population variation in spermatophore volume, number of sperm transferred and sperm length, as well as in volume of the sperm storage organ (spermatheca) and number of tubules, but not in spermatheca length. We found a positive association between sperm number transferred and spermatheca volume. This result suggests that the same postcopulatory mechanisms as in gonochorists drive the correlated evolution of reproductive characters in hermaphrodites.

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INTRODUCTION

Comparative studies in many animal species reveal an extreme diversity in male and female reproductive characters (Eberhard, 1985). Moreover, reproductive traits, e.g. seminal fluid proteins, sperm size or sperm storage organ length, are known to be among the most rapidly evolving types of character (Pitnick et al., 1995, 1999; Swanson & Vacquier, 2002).

Theoretical, comparative and experimental studies suggest that sperm competition, as one aspect of postcopulatory sexual selection, is a principal force in driving the evolution of male ejaculate characteristics (Parker, 1998; Gage & Morrow, 2003; Snook, 2005). Primarily, across a wide range of taxa, the relative numbers of sperm from competing males are positively associated with sperm competition risk or intensity (Harcourt et al., 1981; Gage, 1994; Stockley et al., 1997). Fertilization success during male-male competition is also assumed to be influenced by the size of sperm (Snook, 2005). Sperm length exhibits an enormous variation, both between and within species as well as between individuals (Gage, 1998; Arnaud et al., 2001; Morrow & Gage, 2001); and it seems to evolve rapidly (Pitnick et al., 2003). However, the functional significance of sperm size variability remains poorly understood (Ward, 1998; Snook, 2005). Because the production of long sperm is costly, and there is a trade-off between investment in sperm size and the number of sperm produced (Pitnick, 1996), theory predicts that, when sperm competition is intense, males will produce the smallest sized sperm possible in order to maximize sperm numbers (Parker, 1982). Other results of theoretical models indicate that, unless restrictive conditions are met (cf. Snook, 2005), sperm length is unaffected by differences in sperm competition (Parker, 1998). Accordingly, comparative studies found equivocal results, with either positive (Morrow & Gage, 2000; Byrne et al., 2003), negative (Stockley et al., 1997) or no associations (Hosken, 1997; Gage & Freckleton, 2003) between sperm length and sperm competition risk or intensity.

In internally fertilizing taxa, the competition between sperm of different males takes place within the female reproductive tract. Thus, in addition to sperm competition, the interaction of male ejaculate characteristics with the morphological and physiological environment of the female reproductive tract may generate opportunities for other aspects of postcopulatory sexual selection, i.e. cryptic female choice and sexual conflict (Parker, 1979; Eberhard, 1996). The enormous variation of male and female reproductive

characters might therefore be the result of a correlated evolution, with phenotypic variation in a reproductive trait generating selection on the corresponding trait in the opposite sex (Birkhead & Pizzari, 2002; Miller & Pitnick, 2002). Accumulating evidence for this view comes from comparative studies in taxa as diverse as birds (Briskie & Montgomerie, 1992), beetles (Dybas & Dybas, 1981), butterflies (Gage, 1994), moths (Morrow & Gage, 2000), dung flies (Minder et al., 2005), fruit flies (Pitnick et al., 1999) and stalk-eyed flies (Presgraves et al., 1999), which demonstrate a coevolutionary pattern between sperm length and several characteristics of the female reproductive tract.

Although the majority of studies on sperm-female coevolution have been made across different species, a positive relationship in interacting sex-specific traits can also be found on the intraspecific level. Among different populations of *Drosophila mojavensis*, sperm length and the length of the female spermatheca exhibit a positive association (Pitnick et al., 2003). As sperm morphology and sperm usage by females are central to successful reproduction, their divergence will likely contribute to reproductive isolation among populations and the formation of new species (Parker & Partridge, 1998; Miller & Pitnick, 2002).

Hermaphroditism is widespread in the animal kingdom, and it is now generally accepted that sexual selection applies to all types of gender expression (Morgan, 1994). Nonetheless, hermaphroditic animals are still largely absent from the sexual selection literature (Michiels, 1998). In contrast to species with separate sexes, simultaneous hermaphrodites possess both functional male and female reproductive organs at the same time. Thus, in hermaphrodites selection on traits related to mate acquisition is expected to be intrinsically weaker than in gonochorists (Greeff & Michiels, 1999a), whereas postcopulatory mechanisms might be more important. Individuals of many hermaphroditic species, including gastropods, frequently mate with different partners, resulting in multiply sired-broods (Baur, 1998; Michiels, 1998). Vast numbers of sperm transferred, complex sperm storage organs and mechanisms for the digestion of excess sperm could be an evidence of intersexual interactions, where the evolution of a reproductive trait in the male part triggers an evolutionary response in the female part and vice versa (Tompa, 1984; Michiels, 1998). Previous studies found a great amount of interspecific variation in sperm length in stylommatophoran snails (Tompa, 1984). In addition, a substantial inter- and intraspecific variation in the complex morphology of the female reproductive tract of terrestrial pulmonates, e.g. in the tubule number of the sperm storage organ (spermatheca),

indicates a potential selective pressure acting on male as well as female part of the reproductive tract (Baur, 1998).

In the present study, we used a comparative method to investigate the intraspecific variation in male and female reproductive traits of the simultaneously hermaphroditic land snail *Arianta arbustorum* (L.). The studied traits are assumed to be affected by postcopulatory sexual selection (see Baur 1998). In particular, we quantified the variation in spermatophore volume, number of sperm transferred and sperm length as well as in size of the spermatheca and number of sperm storage tubules and their interrelationships among six natural populations. We also examined whether a male-female interaction influences the divergent evolution of reproductive characters in this hermaphroditic snail species by investigating potential associations between sperm number as well as sperm length with particular female reproductive traits.

MATERIALS AND METHODS

Study organism

Arianta arbustorum is a helicid land snail, common in moist habitats of northern, western and central Europe (Kerney & Cameron, 1979). The snail has determinate growth, individuals become sexually mature at an age of 2–4 years and adults live another 3–4 years (maximum 14 years; Baur & Raboud 1988). Mating includes elaborate courtship with optional dart shooting and lasts 4–12 hours including 1–2 hours copulation (Baur, 1992). Copulation is reciprocal and after simultaneous intromission each snail transfers one spermatophore. The spermatophore (Fig. 1a) has a distinctive form with head filament, sperm container and a 2–3 cm long tail and is deposited in the partner's bursa tract diverticulum (Fig. 1b; Hofmann, 1923). While the vast majority of the received sperm are digested in the bursa copulatrix, a small number escapes into the spermoviduct via the long tail of the spermatophore and travels up to the spermatheca (Lind, 1973). The spermatheca consists of several tubules with a common entrance (Haase & Baur, 1995). In the field, snails deposit 1–3 clutches, each containing 20–50 eggs, per reproductive season (Baur & Raboud, 1988). The mating system of *A. arbustorum* is potentially subject to postcopulatory sexual selection since the snails mate repeatedly during a reproductive season and store viable allosperm for more than one year (Baur, 1988). In addition, sperm precedence (P_2) of double-mated individuals is highly variable (Baur, 1994). Usually,

individuals reproduce through outcrossing, but self-fertilization may occur after being isolated for 2–3 years (Chen, 1994).

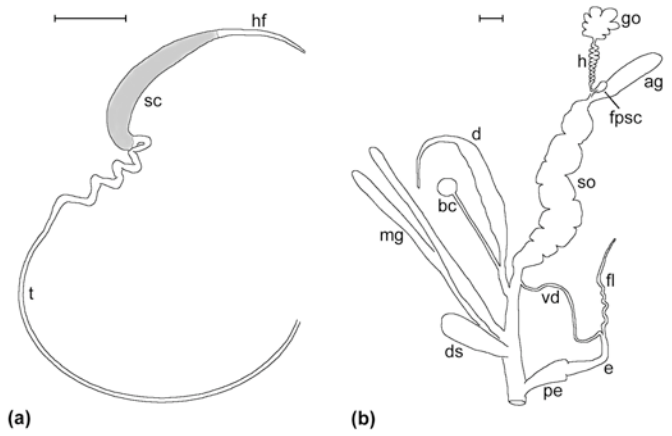

Figure 1. Schematic representation of a spermatophore (a) and the reproductive organs (b) of *Arianta arbustorum*. ag albumen gland, bc bursa copulatrix, d diverticulum, ds dart sac, e epiphallus, fl flagellum, fpsc fertilization pouch-spermatheca complex, go gonad, h hermaphroditic duct, hf head filament of spermatophore, mg mucous glands, pe penis, sc sperm container, so spermoviduct, t tail, vd vas deferens. Scale bars indicate 3 mm.

General methods

Samples of 110–125 subadult *A. arbustorum* were collected between 10 May and 16 July 2003 at each of six different localities in Switzerland (referred to, for convenience, as populations): Nuglar (47°29'N, 7°42'E), Mt. Raimeux (47°19'N, 7°26'E), Gurnigel (46°45'N, 7°27'E), Gantrisch (46°42'N, 7°27'E), Savognin (46°36'N, 9°36'E) and Scuol (46°48'N, 10°18'E). We assume that these populations are isolated, as dispersal in this snail species is expected to be low (e.g. Arter, 1990) and the distance between our studied populations ranged from 4 to 220 km.

Snails were kept singly in transparent plastic beakers (6.5 cm in diameter, 8 cm deep) on moist soil mixed with powdered limestone under a light:dark regime of 16 : 8 h and a constant temperature of 19° ± 1°C. The beakers were cleaned 1–2 times per week, and fresh lettuce was provided *ad libitum* as food. Virgin individuals reached sexual maturity within six weeks as indicated by the formation of a reflected shell lip. For mating, groups of 40–90 snails were placed together in transparent plastic boxes (one box for each population) measuring 34 × 22 × 9.5 cm. All trials were set up in the evening under natural ambient temperature and light conditions and lasted between 12 hours and 5 days. The snails' mating behaviour was checked at intervals of 30–60 min (at night using a torch). When two snails began to court they were separated from the others by placing them in a smaller transparent plastic container. This prevents interference by other snails (cf. Adamo

& Chase, 1990). Snails that did not mate were used in succeeding trials. The frequency of mating was very low and ranged from 0% to 1.4% per test night. In all, 24 trials were run between July and September 2003, resulting in 81 copulations.

We measured shell breadth and height of each mated snail to the nearest 0.1 mm using a vernier calliper and calculated shell volume using the formula: shell volume = $0.312 \times [(\text{shell breadth})^2 \times \text{shell height}] - 0.038$ (B. Baur, unpublished). Shell volume 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. Two hours after copulation one randomly chosen mating partner was killed by decapitation. We removed the shell and dissected the reproductive tract to obtain the spermatophore received from the sperm donor and the spermatheca of the sperm receiver.

Reproductive traits

Digital images of spermatophores were taken to assess the spermatophore volume (expressed as the volume of the sperm-containing part of the spermatophore) using the formula for a truncated cone: $V = \pi L / 12 \times (d_1^2+d_2^2+d_1d_2)$ with L = length along the midline, d_1 = diameter at the small end and d_2 = diameter at the broad end. The number of sperm transferred to the mating partner was assessed by counting the number of sperm heads contained in a spermatophore. The spermatophore consists of a hardened secretion which encapsulates the sperm (Hofmann, 1923). The procedure of sperm counting is described in detail in Locher and Baur (1997). A slight modification of this method was necessary because we also measured the length of sperm from the same spermatophore. In a first step, after having prepared the sperm length slides 12 h after dissection (see below), the spermatophore (still containing a significant amount of sperm) was separated from the sperm suspension and stored at 4°C. The supernatant solution of the sperm suspension was evaporated in an Eppendorf 5301 concentrator at 30°C for 60 min. After replacing the spermatophore to the highly concentrated sperm suspension, 200 μl of phosphate-buffered saline (PBS) was added and the spermatophore was mechanically disrupted for 10–15 min using a pair of microscissors. We stained the sperm heads in this homogenate with an equal volume of a gallocyanin-chromium complex, which binds on the DNA, and subsequently, each sample was treated three times with a sonicator (35 kHz) for 12 h to avoid sperm clusters. Subsamples of known volume were diluted 1 : 1 or 1 : 2 with PBS, depending on the sperm density, and transferred to a Bürker–Türk counting chamber. We counted all sperm heads in randomly chosen cells until the total number of sperm heads

exceeded 400 and used the average of two sub-samples to calculate the total number of sperm in a spermatophore (N), using the formula $N = V \times d \times n/c$ with V = total volume of sample, d = dilution factor, n = number of sperm heads counted in cells and c = volume of counted cells (per cell 25 nl). This technique is accurate and repeatable (Locher & Baur, 1997). The removal of sperm for sperm length measurements corresponds on average to 0.068% of the total number of sperm in a spermatophore (Minoretti & Baur, 2006). Thus, the effect of modifying the sperm counting method is negligible.

To isolate single sperm cells for sperm length measurements without damaging their 800–950 μm long tails, we placed the spermatophore in a drop of Ringer-Solution (cf. Romeis 1989) on a microscopic slide (Minoretti & Baur, 2006). Using insect needles, we opened the sperm containing part along its longitudinal axis. The spermatophore was then placed in 500 μl Ringer solution in a 2 ml Eppendorf tube for 12 h at room temperature. During this period a sufficient proportion of sperm leaves the spermatophore. Each 16 μl of the sperm suspension were put on three microscopic slides and air dried under a glass coverslip. Afterwards, the spermatophore in the sperm suspension was processed for sperm counts (see above). Digital images of sperm cells were obtained at a magnification of ×200. For each snail we measured the total length (head and tail) of 25–30 randomly chosen sperm.

The spermatheca of *A. arbustorum* forms, together with the fertilization chamber, parts of the hermaphroditic duct and spermoviduct, the so-called fertilization pouch-spermatheca complex (FPSC; Tompa 1984). The FPSC of each snail was dissected out from the proximal genital system and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 16 h at room temperature. The length of each sperm storage organ (distance from the branch off of the main tubule to the end of the uppermost tubule) was determined using digital images. The FPSCs of all snails were embedded in paraplast, serially cross-sectioned at 8 μm and stained with haematoxylin-eosin. To estimate the volume available for sperm storage, we measured the area of the lumen of the single tubules on digitized microscopic images and multiplied it with the corresponding thickness of the slices. Volume measurements refer to empty spermathecae, since no sperm had reached the storage organ within 2 h after copulation. This is important, as stored sperm significantly dilates the spermathecal tubules (Bojat & Haase, 2002). The morphological structure of each spermatheca was examined by counting the number of spermathecal tubules.

Image proceeding and statistical analyses

Images of genital traits were obtained using a Sony CCD-Iris camera (Sony, Tokyo, Japan) mounted on a Leica DML light microscope (Leica, Wetzlar, Germany) for sperm length and spermatheca cross-sections or on a Leica MZ 8 binocular for spermatophore volume and spermatheca length assessments. Length and area measurements of digital images were determined using NIH Image public domain software Version 1.63 (http://rsb.info.nih.gov/nih-image). For spermatophore volume and spermatheca length, each measurement was repeated three times and mean values were used for statistical analysis. The reliability of the distance and area measurements was assessed by calculating the intraclass correlation coefficient (Lessells & Boag, 1987) of repeated measurements of subsets. The repeatability for spermatophore size, sperm length as well as spermatheca length and volume ranged from 0.93–0.97, indicating a high accuracy of measurements.

Statistical analyses were performed using SPSS 11 for Mac OS X SPSS Inc., Chicago, IL, USA). The intraspecific variation in reproductive traits was determined using an analysis of covariance (ANCOVA) with shell volume as an indicator for snail size entered as covariate. A Kruskal-Wallis test was used to examine among-population differences in the number of spermathecal tubules because this trait was not normally distributed. Prior to analysis, length data were transformed by raising it to the power of three to have volume measures in all characters. We used the sequential Bonferroni test to control for type I error in multiple comparisons (Rice, 1989).

RESULTS

Intraspecific variation in reproductive traits

Snails from different populations differed significantly in spermatophore volume ($F_{5,69}$ = 28.42, $P < 0.001$; Table 1). A significant part of this variation (68.9%) could be explained by differences among populations. The number of sperm transferred within a spermatophore also varied significantly among populations ($F_{5,69} = 6.70$, $P < 0.001$), with a range from 1.3×10^6 to 3.0×10^6 (population mean values; Table 1). For this trait, 68.5% of the variation was attributable to differences among snails within populations. A correlation revealed a positive association between the volume of the sperm-containing part of the spermatophore and the number of sperm transferred across individuals (r = 0.76, $N = 76$, $P < 0.001$) and also across populations ($r = 0.94$, $N = 6$, $P < 0.01$).

Sperm length differed among the six populations ($F_{5,67} = 25.29$, $P < 0.001$) with a range from 851 μm to 913 μm (population mean values; Table 1). A nested analysis of variance, using the residuals of the relationship between sperm length and snail size, showed that 44.9% of the variation was attributable to differences among populations, 25.7% to differences among snails within populations, and 29.4% to variation among sperm within snails.

Spermatheca length did not differ among populations ($F_{5,65} = 1.49$, $P = 0.20$; Table 1). About 93.1% of the variation in spermatheca length could be explained by differences among snails within populations. In contrast, spermatheca volume varied significantly among populations ($F_{5,65} = 5.08$, $P < 0.01$; Table 1), although 74.1% of this variation was attributable to differences among snails. Morphological examination of the female sperm storage organ revealed two to seven spermathecal tubules. Snails from different populations differed in the number of tubules (Kruskal-Wallis $\chi^2 = 11.07$, $P = 0.05$; Table 1).

Table 1. Summary of shell size, male and female traits of six populations of *Arianta arbustorum*. Data are means ± SE. Sample sizes in parentheses.

	Population (elevation m a.s.l.)					
	Nuglar (430)	Mt Raimeux (1290)	Gurnigel (1320)	Gantrisch (1810)	Savognin (2200)	Scuol (2250)
Shell volume (cm^3)	1.89 ± 0.07 (14)	1.52 ± 0.03 (22)	1.33 ± 0.02 (42)	1.28 ± 0.03 (32)	1.83 ± 0.03 (28)	2.04 ± 0.04 (24)
Male traits						
Spermatophore volume (mm^3)	2.96 ± 0.39 (5)	4.13 ± 0.20 (11)	1.79 ± 0.11 (19)	2.15 ± 0.13 (16)	2.40 ± 0.14 (13)	2.06 ± 0.14 (12)
Number of sperm ($\times 10^6$)	2.46 ± 0.49 (5)	3.04 ± 0.26 (11)	1.34 ± 0.16 (19)	1.37 ± 0.19 (16)	1.95 ± 0.15 (13)	1.92 ± 0.24 (12)
Sperm length (μm)	913 ± 10 (6)	900 ± 4 (11)	854 ± 3 (17)	859 ± 4 (15)	860 ± 3 (13)	851 ± 7 (12)
Female traits						
Spermatheca length (mm)	2.67 ± 0.13 (7)	2.50 ± 0.05 (10)	2.24 ± 0.05 (20)	2.44 ± 0.06 (14)	2.62 ± 0.06 (13)	2.78 ± 0.17 (8)
Spermatheca volume ($\times 10^{-3} mm^3$)	2.48 ± 0.34 (7)	2.87 ± 0.32 (10)	1.63 ± 0.14 (18)	1.32 ± 0.12 (13)	2.51 ± 0.20 (12)	2.74 ± 0.29 (12)
Tubule number	5.6 ± 0.4 (7)	4.4 ± 0.3 (10)	4.6 ± 0.2 (20)	4.7 ± 0.3 (13)	3.9 ± 0.3 (13)	4.6 ± 0.3 (12)

Allometric relationships

Regression analyses were used to examine potential relationships between snail size and male as well as female traits. We found no significant relationships between snail size and spermatophore volume ($r^2 = 0.01$, $F_{1,74} = 0.35$, $P = 0.56$), number of sperm transferred ($r^2 = 0.02$, $F_{1,74} = 1.85$, $P = 0.18$) or sperm length ($r^2 = 0.02$, $F_{1,72} = 1.24$, $P = 0.27$). However, there was a significant positive relationship between snail size and spermatheca length ($r^2 = 0.30$, $F_{1,70} = 30.11$, $P < 0.001$) as well as spermatheca volume ($r^2 = 0.16$, $F_{1,70} = 12.91$, $P < 0.01$). Therefore, we controlled for these allometric relationships by calculating residuals from regressions for subsequent statistical analysis. The number of spermathecal tubules was not correlated with snail size (Spearman rank correlation $r_s = -0.11$, $N = 75$, $P = 0.36$).

Reproductive character associations

Due to the inter-correlation between spermatophore volume and number of sperm transferred we only considered sperm number and sperm length as male traits in further analyses.

At the population level, we found a positive relationship between the number of sperm transferred and the residual spermatheca volume ($r^2 = 0.77$, $F_{1,4} = 13.26$, $P < 0.05$; Fig. 2). However, the mean number of sperm transferred showed no significant association with the mean number of female sperm storage tubules, as an indicator for the complexity of the sperm storage organ (Spearman rank correlation $r_s = –0.26$, $N = 6$, $P = 0.31$).

Mean population sperm length was not significantly related to residual mean population spermatheca length ($r^2 = 0.07$, $F_{1,4} = 0.28$, $P = 0.62$) and residual mean population spermatheca volume ($r^2 = 0.20$, $F_{1,4} = 0.99$, $P = 0.38$).

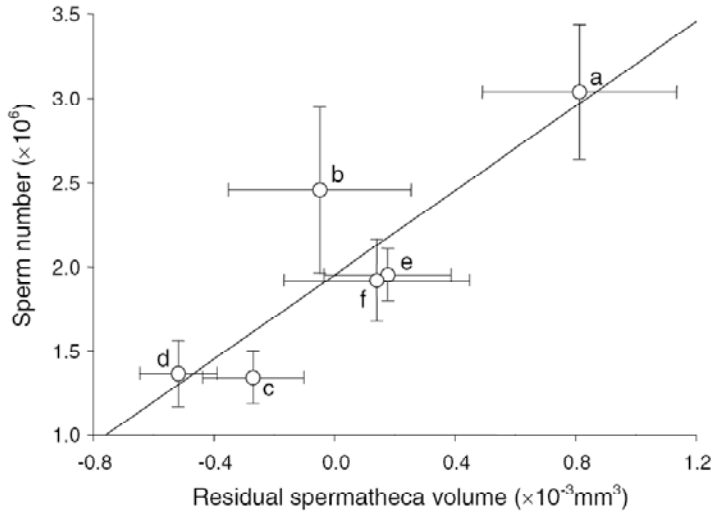

Figure 2. Relationship between mean population sperm number and residual mean population spermatheca volume. Characters indicate populations: a, Nuglar; b, Mt. Raimeux; c, Gurnigel; d, Gantrisch; e, Savognin; f, Scuol. Bars indicate s.e.

Parker (1982) predicted that sperm competition should select for the evolution of numerous, tiny sperm. We tested this prediction by examining the relationship between the number of sperm transferred and sperm length. Interestingly, we found a positive relationship between sperm number and sperm length across individual snails ($r = 0.57$, $N = 73$, $P < 0.001$) and a marginally significant relationship across populations ($r = 0.81$, $N = 6$, $P = 0.052$).

DISCUSSION

The main result of our study showed that the number of sperm transferred is positively associated with the volume of the female sperm storage organ in *Arianta arbustorum*. Evidence for coevolution between the number of sperm produced (measured as testis size) and spermathecal volume has already been found in insects (Morrow & Gage, 2000; Minder et al., 2005). However, to our knowledge, the present results are the first supportive evidence of an evolutionary association between an ejaculate characteristic and a female reproductive trait in a simultaneously hermaphroditic animal.

There are several hypotheses that may explain this coevolutionary pattern. First, a role for pleiotropy in generating a correlated divergence of reproductive traits cannot be ruled out (Arnqvist & Thornhill, 1998). Nevertheless, we hardly expect pleiotropy between the size of a female-specific somatic organ and the number of male-specific sex cells because of the origin from different cell lines. Furthermore, both sexes could evolve in response to a common natural selection regime, e.g. parasite pressure, predator avoidance or food acquisition, resulting in a correlated evolution (Arnqvist & Rowe, 2002). The association between sperm number and spermatheca volume may also simply be fertility driven (i.e. the more eggs the female function produces, the more sperm are required). Therefore, an increase in the volume of the sperm stores may exert selection upon males to evolve greater ejaculate masses to fill the spermatheca (Minder et al., 2005). This could also explain the allometric association between snail size and spermatheca size. Additionally, traditional models of sexual selection may influence the divergence of reproductive characters. For instance, a linkage disequilibrium between female preference and male ornament, which is consistent with sexy sons (Runaway or Fisherian selection) or good genes models (Hosken & Stockley, 2004), or 'sperm choice' by females could affect reproductive trait evolution (Birkhead, 1998; Miller & Pitnick, 2002). However, female choice mechanisms seem rather to select for sperm quality traits than for sperm numbers.

More likely, however, the positive correlation between sperm number and the volume of the sperm storage organ is the result of an antagonistic coevolution driven by a conflict between mating partners over the processes involved in sperm storage (Rice, 1996; Gavrilets, 2000). There is accumulating evidence that intersexual conflicts can play an important role in generating a rapid evolution of reproductive characters that increase reproductive success in one sex even when they are costly to the other sex (e.g. Hosken et al., 2001; Arnqvist & Rowe, 2002). Selection for increased sperm numbers may arise

because of the advantage it gives to males as a guard against sperm competition from future males, rather than as a response that enhances success during sperm competition (Simmons & Siva-Jothy, 1998). Males that produce enough sperm to completely fill the female's storage organ may be favoured, because they prevent the access of sperm from subsequent mating partners (Retnakaran, 1974). As this male strategy aims to prevent either sperm competition or females from re-mating, it may lead to a conflict by reducing female opportunities to gain fitness benefits from multiple mating (Stockley, 1997). Although in *A. arbustorum* one copulation would be sufficient to fertilize all eggs produced in one year (Chen & Baur, 1993), multiple mating may benefit individuals by increasing the fitness of the offspring like in species with separate sexes, e.g. by 'trading up' to better-quality mates, increasing the genetic quality of offspring or avoiding the use of sperm from genetically incompatible partners (Kempenaers et al., 1992; Tregenza & Wedell, 1998; Baer & Schmid-Hempel, 1999). Consistent with the spermathecal filling hypothesis (Simmons & Siva-Jothy, 1998), the conflict over the control of fertilization decisions may potentially set off an evolutionary arms race between the number of sperm transferred and spermatheca volume.

In simultaneous hermaphrodites, intersexual conflicts seem to be pronounced because individuals act both, as males and as females during mating (Charnov, 1979; Michiels, 1998). The antagonistic reproductive interests of both components are therefore assumed to account for the evolution of special adaptations. For example, rather than avoiding being inseminated, individuals of many snail species digest received sperm (Lind, 1973), which could influence the outcome of postcopulatory sexual selection with respect to ejaculate traits and sperm storage organ size. Sperm digestion reduces the competitive ability of an ejaculate (Greeff & Michiels, 1999b) and thus, should select for increased sperm numbers as well as structures to protect sperm against digestion. Indeed, many snail species transfer high numbers of sperm packed in a spermatophore (Baur, 1998), but even so only a small fraction escapes the digesting bursa copulatrix of the receiver (0.1% in *Helix pomatia*, Lind 1973; 0.025% in *H. aspersa*, Rogers & Chase 2001). However, as data from individuals of *A. arbustorum* collected in the field demonstrate, still a sufficient amount of sperm may reach the spermatheca (Baminger & Haase, 1999).

The variation in the number of spermathecal tubules suggests possibilities of the female reproductive tract to control fertilization (Haase & Baur, 1995; Hellriegel & Ward, 1998). A larger number of tubules may allow a better separation of sperm from different mating partners and later also a selective use of sperm for fertilization, favouring a

particular partner (Baur, 1998; Hellriegel & Ward, 1998). Although coevolution of ejaculate traits and sperm storage organ complexity could arise through runaway processes driven by female choice (Keller & Reeve, 1995) or conflicts between the sex functions over sperm use (Rice & Holland, 1997; Stockley, 1997), there was no association between the number of sperm transferred and tubule number in our study species.

Contrary to Parker's prediction of a trade-off in sperm investment (Parker, 1982), we found a positive relationship between sperm number and length in *A. arbustorum*. Large sperm may function in blocking the female's sperm stores to future males (Ladle & Foster, 1992; Briskie et al., 1997) or prevent sperm from being displaced from storage once they have gained entry (Sivinski, 1980; Dybas & Dybas, 1981). Sperm in *A. arbustorum* and some other snail species are usually stored at the bulbous blind ends of the tubules of the spermatheca, with their heads in tight contact with the spermathecal epithelium (Bojat et al., 2001; Rogers & Chase, 2002). Rogers & Chase (2002) suggested that the unified beating of the flagella of sperm from the first mating should provide paternity assurance through increased resistance to incoming sperm from subsequent matings entering the tubules; the higher the number of sperm, the stronger the resistive force. Thus, the number of sperm transferred and sperm length potentially act together in the competition of preventing rival sperm gaining access to the sperm stores in order to increase fertilization success. Furthermore, if large sperm have a selective advantage in sperm competition, e.g. by a better ability to block the storage organ and thereby prevent rival sperm from gaining access or by a better ability to resist displacement, a coevolution between sperm morphology and female reproductive tract morphology is predicted (Pitnick et al., 1999; Simmons, 2001). However, in contrast to comparative studies in gonochorists (Briskie & Montgomerie, 1992; Briskie et al., 1997; Presgraves et al., 1999; Pitnick et al., 2003), sperm length in *A. arbustorum* was not associated with spermatheca size.

Perpetual changes in interacting reproductive characters may lead to a divergence among populations because selection is acting directly on traits that affect the reproductive success of individuals (Panhuis et al., 2001). Thus, with restricted gene flow among populations, coevolution of reproductive traits may constitute an important and widespread source of reproductive incompatibility of individuals from different populations (Parker & Partridge, 1998; Gavrilets, 2000). According to theory, the expected outcome may be rapid genetic divergence and eventual speciation, particularly in large populations (Gavrilets, 2000), and there is comparative as well as experimental evidence supporting this idea (Arnqvist et al., 2000; Martin & Hosken, 2003). As we studied snails from populations

with presumably no gene exchange, the male and female reproductive character divergence in *A. arbustorum* might possibly contribute to reproductive isolation among these populations.

The different evolutionary scenarios for male-female coevolution discussed above need not to be mutually exclusive. There are a number of potential explanations for correlations between morphological traits, and although comparative studies are critical in detecting correlated evolution, reliable inferences about underlying mechanisms cannot be definitely made based on such patterns alone (Arnqvist & Rowe, 2002; Hosken & Stockley, 2004). Nevertheless, our study suggests similar mechanisms driving the coevolutionary changes in reproductive characters in gonochoristic and hermaphroditic animals. Future studies will show whether the observed pattern of correlated evolution of sex-specific traits in *A. arbustorum* reflects a general pattern in simultaneous hermaphrodites.

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CHAPTER III

EXPANDABLE SPERMATHECA INFLUENCES SPERM STORAGE IN THE SIMULTANEOUSLY HERMAPHRODITIC LAND SNAIL *ARIANTA ARBUSTORUM*

Kathleen Beese & Bruno Baur

ABSTRACT

In many simultaneously hermaphroditic land snail species, the sperm storage organ (spermatheca) is highly structured, suggesting that the female function might be able to influence offspring paternity. Physical properties of the sperm storage organ, including its initial size and sperm storage capacity, may also affect fertilization patterns in multiply mated snails. We examined the structure, volume and tubule length of empty spermathecae in the land snail, *Arianta arbustorum*, and assessed differences in spermatheca size following a single copulation. The number of spermathecal tubules ranged from 2–7, but was not correlated with the volume of empty spermathecae. The volume of sperm stored in the spermatheca after a copulation was correlated with neither the number of spermathecal tubules nor copulation duration. Mean spermathecal volume more than doubled between two and thirty-six hours after sperm uptake, but the length of the spermathecal tubules did not change. Interestingly, the volume of sperm stored in the spermatheca seems not to be related to the size of the spermatophore and thus not to the number of sperm received (=allosperm). The amount of allosperm digested in the bursa copulatrix was highly variable and no significant relationship with the size of the spermatophore received was found. These findings suggest that numerical aspects of sperm transfer are less important in influencing fertilization success of sperm in *A. arbustorum* than properties of the female reproductive tract of the sperm receiver.

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INTRODUCTION

In internally fertilizing species, mating with multiple partners and long-term storage of sperm can lead to intersexual conflicts over the postcopulatory processes involved in sperm storage and sperm use (Arnqvist & Rowe, 2005). The sperm donor may develop strategies that increase the ability to outcompete the ejaculates of rival mates inside the sperm receiver and thus to enhance its own fertilization success. For its part, the sperm receiver, which may directly and indirectly benefit from multiple matings (e.g. Jennions and Petrie, 2000), should respond to the restrictions of polyandry with adaptations to allow for postcopulatory mate choice to be retained. Adaptations of the sperm receiver to exert control over paternity may include the selective storage of sperm in complex storage organs (Hellriegel & Ward, 1998; Pitnick et al., 1999) and the selective extrusion or digestion of sperm, thereby reducing the amount of sperm reaching the storage organ (Eberhard, 1985; Birkhead et al., 1993).

Highly structured sperm storage organs occur in species of several animal groups, e.g. in millipedes (Barnett et al., 1995), spiders and flies (Eberhard, 1985; Eberhard, 1996), lizards (Fox, 1963) and birds (Briskie, 1996). The spatial separation of sperm from multiple males in different spermathecal compartments has been documented in the yellow dung fly (e.g. Hellriegel and Bernasconi, 2000). The shape of the sperm storage organ (spherical or tubular) and the variation in physical properties (e.g. elasticity) may also influence sperm utilization (Walker, 1980; Simmons, 2001). For example, sperm stratification in the tubular spermatheca of red flour beetles influences fertilization success (Lewis et al., 2005), whereas the expandable spermatheca in some gryllids can accommodate successive ejaculates, resulting in random utilization of sperm (Simmons, 1986).

Recently, it has been recognized that sexual selection (including sexual conflict) is also an important factor in the evolution of reproductive traits in simultaneous hermaphrodites, where both sexes are combined within one individual (Charnov, 1979; Michiels, 1998). As in species with separate sexes, multiple matings and long-term sperm storage are common in hermaphrodites. Consequently, strategies of the sperm receiver to influence fertilization may also exist (Baur, 1998; Michiels, 1998). In terrestrial pulmonate snails, which are all simultaneous hermaphrodites, the spermatheca can be highly structured and there is considerable among- and within-species variation in the number of

sperm storing tubules (Baur, 1998). Furthermore, the female reproductive tract of land snails is extremely hostile to sperm received (=allosperm). Compared with the number of sperm received, the proportion of allosperm stored is very low (Lind, 1973; Rogers & Chase, 2001). The remaining sperm are digested in a specialized organ called the bursa copulatrix.

In the helicid land snail, *Arianta arbustorum* (Linnaeus, 1758), a controlled experiment with double-mated individuals revealed a considerable variation in sperm utilization patterns (Baur, 1994a). This variation may be due to differences among sperm donors, e.g. in the amount of sperm transferred and/or sperm quality, or be influenced by the morphology and hostility of the sperm receiver's reproductive tract.

The aim of the present study was to examine the potential importance of complexity, shape and physical properties of the sperm receiver's spermatheca for paternity in *A. arbustorum*. This gastropod is common in moist habitats of northern, western and central Europe (Kerney & Cameron, 1979). The snail has determinate growth, with individuals becoming sexually mature at an age of 2–4 years and adults living another 3–4 years (maximum 14 years; Baur and Raboud, 1988). The snails mate repeatedly during a reproductive season and store viable allosperm for more than one year (Baur, 1988). Individuals usually reproduce through outcrossing, but self-fertilization may occur after isolation for 2–3 years (Chen, 1994). Mating includes elaborate courtship with optional dart shooting and lasts 2–18 h (Hofmann, 1923). Copulation is reciprocal and after simultaneous intromission each snail transfers one spermatophore, consisting of a hardened secretion that encapsulates the sperm, into the partner's bursa tract diverticulum (Hofmann, 1923). Previous studies showed that mating partners do not adjust the number of sperm transferred (Baur et al., 1998). Furthermore, the number of sperm transferred is unaffected by copulation duration, degree of sperm competition risk or nutritional stress (Locher and Baur 1999, 2000, 2002). Three to four weeks after successful copulations, *A. arbustorum* has entirely replenished its autosperm reserves (Hänggi et al., 2002). In addition, no direct effects of dart-shooting on sperm transfer and sperm storage have been found (Baminger et al., 2000; Bojat & Haase, 2002).

Allosperm received reach the spermoviduct via the spermatophore's tail and travel up to the spermatheca, where they are stored (Lind, 1973). However, the vast majority of sperm received is transported to the sperm-digesting bursa copulatrix. The spermatheca of *A. arbustorum* consists of 2–9 blind-ended tubules uniting to a common duct, which opens into the fertilization pouch (e.g. Baminger et al., 2000). Morphological studies have shown

that the stored allosperm are not equally distributed among all spermathecal tubules, suggesting that sperm from different mates might be both stored and used separately (Haase & Baur, 1995; Baminger & Haase, 1999; Baminger et al., 2000; Bojat & Haase, 2002). Furthermore, ultrastructural analyses indicate that ciliation of the spermathecal epithelium and musculature surrounding the spermathecal tubules may allow manipulations of sperm storage and release (Bojat et al. 2001a, 2001b). So far, however, the physical properties of the spermatheca, such as its initial size and sperm storage capacity have not been investigated. We therefore examined the volume and tubule length of empty spermathecae in relation to the variable spermatheca structure. We also assessed changes in spermatheca size (volume, tubule length) after the uptake of sperm following a single copulation and examined whether copulation duration influenced the amount of sperm stored. The relationship between sperm transfer and sperm use was estimated by assessing the assumed initial size of the received spermatophore and number of sperm still confined in the spermatophore and comparing these with the volume of allosperm stored in the spermatheca and the volume of the sperm digesting bursa copulatrix.

MATERIALS AND METHODS

A sample of 120 sub-adult specimens of *Arianta arbustorum* was collected in a subalpine forest near Gurnigelbad, 30 km south of Bern, Switzerland (46°45'N, 7°27'E) on 10 May 2003. Snails were raised individually to adulthood in transparent plastic beakers (6.5 cm in diameter, 8 cm deep) on moist soil mixed with powdered limestone. The snails were kept under a light:dark regime of 16 : 8 h and a constant temperature of 19 ± 1°C. The beakers were cleaned 1–2 times per week, and fresh lettuce was provided *ad libitum* as food. Sexual maturity, as indicated by the formation of a reflected shell lip, was attained within 6 weeks.

Mating trials were set up at night under natural ambient temperature and light conditions. Two groups each of 60 snails were placed together in transparent plastic boxes (34 × 22 × 9.5 cm) in which snails were allowed to court. The snail's mating behaviour was checked at intervals of 15–30 min (at night using a torch). When two snails began to court they were separated from the others by placing them in a smaller transparent plastic container. This prevents interference by other snails (cf. Adamo and Chase, 1990). Copulating snails were observed at intervals of 15 min. We recorded the courtship duration for each mating pair. Snails that did not mate were used in succeeding trials.

We assumed that, after reception, the spermatophore has been moved into the final position 2 h after copulation (cf. Baumgartner 1997) and that sperm migration from the spermatophore to the spermatheca had ceased after 36 h (cf. Lind, 1973; Bojat and Haase, 2002). Consequently, one randomly chosen mating partner was killed by decapitation 2 h after copulation, the other mating partner 36 h after copulation. Prior to dissection, shell breadth and height of each mated snail were measured to the nearest 0.1 mm using a vernier calliper. Shell volume was calculated using the formula: $V = 0.312 \times [(\text{breadth})^2 \times \text{height}] - 0.038$ (B. Baur, unpublished). Shell volume is a more reliable measurement of snail size than weight because weight depends on the state of hydration and thus is highly variable in terrestrial gastropods.

Together with the fertilization pouch, parts of the spermoviduct and hermaphroditic duct, the spermatheca forms the so-called fertilization pouch-spermatheca complex (FPSC; Tompa, 1984; see insert in Fig. 3). FPSC's of all mated snails were embedded in paraplast, serially cross-sectioned at 8 μm and stained with haematoxylin-eosin. For each mated snail, the structure of the spermatheca was examined by counting the number of spermathecal tubules. The length of each tubule was approximated by counting the number of cross-sections (cf. Cuellar, 1966), starting with that section in which a tubule was clearly separated from the tubule from which it branched off. The main tubule of the spermatheca was always the longest tubule. For each tubule a semi-quantitative assessment of the amount of allosperm stored was made using the following classes: no sperm found, sporadic sperm occurrence, sperm loosely packed, sperm densely packed. We estimated the spermatheca volume by measuring the area of the lumen on digitized microscopic images and multiplying it with the corresponding thickness of the slices. The volume of allosperm stored in the spermatheca was measured in the same way. Spermatheca volume and the volume of allosperm stored are expressed as the sum of single tubule measurements.

The spermatophores were dissected from the diverticulum and digital images were taken to assess spermatophore volume [expressed as the volume of the sperm-containing part of the spermatophore; see Beese et al. (2006) for an illustration of a spermatophore]. We calculated the volume by using the formula for a truncated cone: $V = \pi L / 12 \times (d_1^2+d_2^2+d_1d_2)$ with L = length along the midline, d_1 = diameter at the narrow end, and d_2 = diameter at the broad end. To assess the assumed initial spermatophore volume of snails 36 h after copulation we multiplied the obtained volume with a factor of 1.33. This factor was

obtained by dividing the mean spermatophore volume 2 h after copulation through the mean spermatophore volume 36 h after copulation.

The number of sperm transferred to the mating partner was assessed by counting the number of sperm heads in a spermatophore (Locher & Baur, 1997). The spermatophore was mechanically disrupted in 200 μl phosphate-buffered saline (PBS) for 10–15 min using a pair of microscissors. Subsequently, we added an equal volume of a gallocyanin-chromium complex to the homogenate to stain the sperm heads (Einarson, 1951). Each sample was treated three times with a sonicator (35 kHz) for 12 h to separate sperm clusters. Subsamples of known volume were diluted 1 : 1 or 1 : 2 with PBS, depending on the sperm density, and transferred to a Bürker–Türk counting chamber. We counted all sperm heads in randomly chosen cells until the total number of sperm heads exceeded 400 and used the average of two sub-samples to calculate the total number of sperm in a spermatophore. This technique is accurate and repeatable (Locher & Baur, 1997).

The bursa copulatrix volume was assessed on digital images and calculated by using the formula for an ellipsoid body: $V = 4/3\pi \times L/2 \times (W/2)^2$ with L = length axis diameter and W = width axis diameter.

Images were obtained using a Sony CCD-Iris camera mounted on a Leica DML compound microscope for spermatheca cross-sections or on a Leica MZ 8 binocular microscope for spermatophore volume and bursa copulatrix volume. Length and area measurements of digital images were determined using NIH Image public domain software Version 1.63 (http://rsb.info.nih.gov/nih-image). For spermatophore volume and bursa copulatrix volume, each measurement was repeated three times and means were used for statistics. The repeatability of measurements was assessed following Lessells and Boag (1987) and ranged from 0.93–0.97, indicating a high accuracy.

Means ± SD are given unless otherwise stated. Statistical analyses were performed using SPSS 11 for Mac OS X. Possible differences in reproductive traits were determined using analyses of covariance (ANCOVA, type III model) with time after mating (2 h or 36 h) as factor and shell volume (an indicator of snail size) as covariate. In no analysis did shell volume have any significant influence, and we therefore removed it from the analyses and presented the results of one-way ANOVAs with time after mating as factor. Prior to analysis, length data were transformed by raising them to the power of three to have volume measures in all characters. The power of r (alpha = 0.05) for non-significant correlations was calculated following Cohen (1988). Spearman rank correlations were used

to examine possible relationships between the number of spermathecal tubules and reproductive traits. In two individuals, no allosperm were stored after 36 h. These animals were not considered in the data analyses.

RESULTS

Spermatheca size in relation to sperm storage

The volume of empty spermathecae (2 h after mating) ranged from 0.68×10^{-3} to 3.04×10^{-3} mm^3 (Table 1). Cumulative tubule length varied between 2.38 and 5.23 mm and main tubule length between 1.11 and 2.14 mm. The number of spermathecal tubules ranged from 2 to 7 (median 4.5). The volume of empty spermathecae, cumulative tubule length, main tubule length and tubule number were not found to be correlated with shell size (in all cases $P > 0.15$). Furthermore, we found no correlation between the volume of empty spermathecae and the number of tubules ($r_s = 0.20$, $N = 18$, $P = 0.42$).

Table 1. Summary of shell size, male and female traits of six populations of *Arianta arbustorum*. Data are means ± s.e. Sample sizes in parentheses.

	Time after copulation		*df*	*F*	*P*
	2 h	36 h			
Spermatheca volume ($\times 10^{-3}$ mm^3)	1.62 ± 0.61 (18)	3.80 ± 1.96 (18)	1, 34	20.16	<0.001
Cumulative tubule length (mm)	3.20 ± 0.78 (19)	3.34 ± 0.84 (18)	1, 35	0.28	0.598
Main tubule length (mm)	1.54 ± 0.32 (19)	1.63 ± 0.29 (18)	1, 35	0.66	0.421
Volume of spermatophore transferred (mm^3)	1.79 ± 0.50 (19)	1.35 ± 0.60 (17)	1, 34	5.29	0.028
Sperm content of spermatophore (number of sperm $\times 10^6$)	1.34 ± 0.68 (19)	0.25 ± 0.34 (17)	1, 34	36.99	<0.001

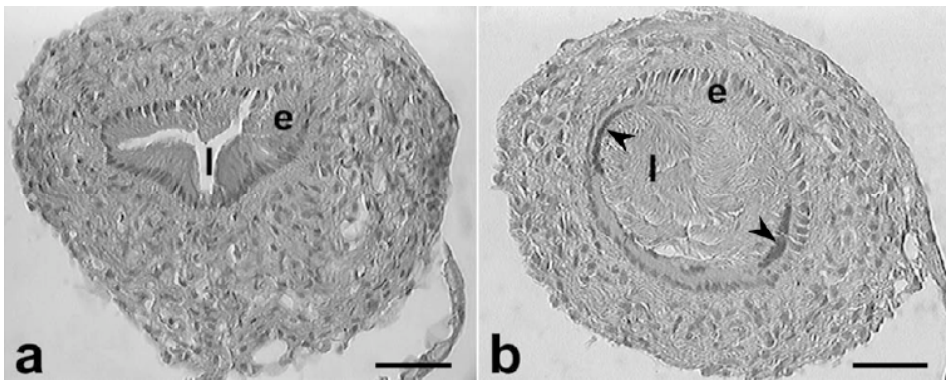

Figure 1. Cross-section through the proximal end of the main spermathecal tubule of a) an empty spermatheca (2 h after copulation), and b) a spermatheca filled with sperm (36 h after copulation; sperm heads indicated by arrowheads). e, epithelium; l, lumen of main tubule. Scale bar = 50 μm.

The storage of allosperm resulted in an increase in spermatheca volume (Fig. 1; Table 1). The volume of sperm stored in the spermatheca 36 h after copulation averaged $1.78 \times 10^{-3} \pm 1.63 \times 10^{-3}$ mm^3 ($N = 18$; range: 0.02×10^{-3}–4.47×10^{-3} mm^3). The volume of sperm stored and the volume of the spermatheca were positively correlated ($r = 0.95$, $N = 18$, $P < 0.001$; Fig. 2). However, cumulative length of spermathecal tubules and the length of the main tubule did not differ before and after sperm storage (Table 1). The volume of the spermatheca 36 h after copulation ($r_s = -0.31$, $N = 18$, $P = 0.21$) and the volume of sperm stored ($r_s = -0.42$, $N = 18$, $P = 0.09$) were not correlated with the number of spermathecal tubules. These results indicate that the volume of sperm stored from a single spermatophore is not influenced by the number of spermathecal tubules. Spermatheca volume and volume of sperm stored in the spermathecae were correlated with neither the shell size of the sperm receiver nor with that of the sperm donor (in all cases $P > 0.35$).

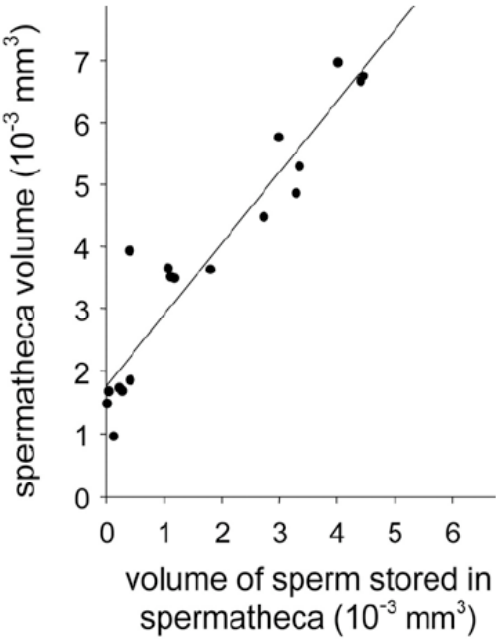

Figure 2. Correlation between the volume of sperm stored in the spermatheca 36 h after copulation and spermatheca volume in 18 individuals of *Arianta arbustorum*.

As in previous studies (Baminger & Haase, 1999; Baminger & Haase, 2000; Bojat & Haase, 2002), most of the allosperm were stored in the main spermathecal tubule (Fig. 3). The amount of sperm stored in lateral tubules was variable. In five out of 18 individuals all lateral tubules were empty. In the remaining snails one or more lateral tubules and in four individuals all lateral tubules contained at least a few sperm. In most snails, the first lateral tubules contained more allosperm than the remaining lateral tubules.

The volume of sperm stored in the spermatheca was not influenced by copulation duration (median: 97.5 min, range: 65–135 min; $r_s = -0.05$, $N = 18$, $P = 0.83$).

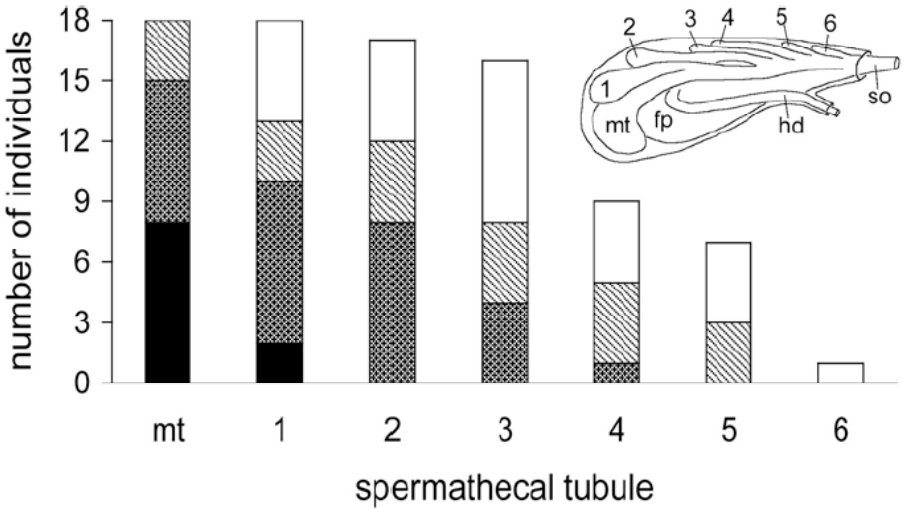

Figure 3. Frequency distribution of the amount of sperm stored in the spermathecal tubules of *Arianta arbustorum* (mt = main tubule, 1–6 = lateral tubules; see insert). The amount of sperm is indicated as sperm densily packed (black), sperm loosely packed (chequered), sporadic sperm occurrence (diagonally hatched) and no sperm found (white). The insert shows a schematic representation of the fertilization pouch-spermatheca complex (fp, fertilization pouch; hd, hermaphroditic duct; so, spermoviduct).

Spermatophore volume and sperm number

The spermatophore volume and the number of sperm it contained 2 h after copulation were positively correlated ($r = 0.80$, $N = 19$, $P < 0.001$). Both spermatophore volume and the number of sperm it contained differed between snails examined 2 h and 36 h after mating (Table 1). The volume of full spermatophores (2 h after mating) and the number of sperm transferred were correlated with neither the shell size of the sperm donor, nor that of the sperm receiver (in all cases $P > 0.10$).

The transfer of larger spermatophores did not result in more sperm reaching the storage organ 36 h after mating ($r = 0.35$, $N = 14$, $P = 0.22$), assuming that the spermatophore volume decreased to the same degree in all individuals. Furthermore, if a larger number of sperm delivered also results in a larger number of sperm stored, we would expect a negative correlation between the amount of sperm remaining in the spermatophore 36 h after mating and the volume of sperm stored in the spermatheca. There was no correlation between these two traits ($r = 0.03$, $N = 14$, $P = 0.92$). However, in both correlations the statistical power was too low to provide sufficient evidence that there is no effect (power of 0.25 and 0.02, respectively).

Assuming that a fixed proportion of sperm is digested, then the transfer of large spermatophores with larger numbers of sperm should result in more sperm being transferred into the digesting bursa copulatrix. Furthermore, the less sperm that remain in

the spermatophore the more should be found in the bursa. Indeed, we found a positive correlation between spermatophore volume and bursa copulatrix volume 36 h after mating ($r = 0.67$, $N = 9$, $P = 0.049$), but also between number of sperm remaining in the spermatophore and bursa volume ($r = 0.67$, $N = 9$, $P = 0.050$). However, after removing a single outlier, both correlations were no longer significant ($P > 0.58$).

The amount of sperm reaching the spermatheca was very low, corresponding to only 0.09% of the amount of sperm received (based on mean spermatophore volume 2 h after copulation, $N = 16$). However, this is a very rough estimate because the spermatheca shrinks during processing for histology.

DISCUSSION

Our study shows that the spermatheca of the helicid land snail, *Arianta arbustorum*, is expandable and can accommodate more sperm than would be expected from measuring its initial volume. After a single copulation, 45% of the spermatheca volume was filled with sperm. The volume of sperm stored in the spermatheca 36 h after copulation corresponded to 98% of the volume of empty spermathecae. The increase in spermatheca volume must solely be due to the expansion of tubule diameter, because sperm uptake did not influence the length of the spermathecal tubules. Histological investigations revealed that the increase in spermathecal volume may be associated with stretching of the spermathecal epithelium (Beese, pers. obs.), which possesses extensive interdigitations between lateral and basal cell membranes in the unfilled state (Bojat et al., 2001b).

Our results confirmed the morphological structure of the spermatheca previously described (Haase & Baur, 1995; Baminger & Haase, 1999; Bojat & Haase, 2002). However, in contrast to Bojat and Haase (2002), we found no correlation between tubule number and amount of sperm stored. Furthermore, tubule number did not influence the volume of empty spermathecae, which contradicts the assumption of Bojat and Haase (2002).

Consistent with previous studies, there are indications that the spermathecal tubules are filled with sperm in a certain sequence, starting with the main tubule and continuing with the first lateral tubule, followed by the second lateral tubule and so on (Baminger & Haase, 1999). This indicates a potential for spatially separating sperm from multiple mates in different tubules and thus may represent a mechanism for cryptic mate choice. However, the tubular structure of the spermatheca could also be a naturally selected adaptation for

efficient sperm storage and use. The nutrition of spermatozoa might be an important function of the spermathecal epithelium in *A. arbustorum* and the observed sperm storage pattern may be the result of sperm being nourished during storage (Bojat et al., 2003). Sperm are stored with their heads in tight contact with the spermathecal epithelium, usually at the bulbous blind tubule ends (Bojat et al., 2001b). The tails of the spermatozoa beat in synchronous waves that are directed towards the entrance of the tubules (Rogers & Chase, 2002). The activity of sperm during storage and the long periods during which sperm remain viable suggest that sperm may require energy in the spermatheca. Long tubules provide a large surface for sperm attachment and, potentially, for nourishment. However, an uptake of nutrients by stored sperm has not yet been demonstrated in *A. arbustorum* (Bojat et al., 2001b) and tubular spermathecae might have evolved simply to reduce sperm loss, as suggested for the red flour beetle (Fedina & Lewis, 2004), or to match storage capacity demands arising through divergence in female longevity or egg productivity (Pitnick et al., 1999).

Spermatheca shape and capacity potentially influence sperm utilization patterns (Walker, 1980; Simmons, 2001). A tubular structure, as found in *A. arbustorum* and other snail species, may lead to sperm stratification and thereby result in last-mate sperm precedence. However, there is no evidence for sperm stratification nor for consistent last-mate sperm precedence in helicid snails (Baur, 1994a; Rogers & Chase, 2002; Evanno et al., 2005). On the contrary, first-mate sperm precedence has been found in the first brood of individuals of *A. arbustorum* that mated twice within 70 days (Baur, 1994a) and also in *Helix aspersa* (Evanno et al., 2005). The observed pattern could be due to the spermatheca being filled to capacity during the first mating (Retnakaran, 1974), but it might also be explained by the activity of allosperm during storage. Rogers and Chase (2002) suggested that the beating of the flagella of sperm from the first mate may generate resistance to incoming sperm from subsequent mates; the higher the number of resident sperm, the stronger the resistive force. Thus, the probability of sperm gaining access to the spermathecal tubules would decrease with each successive mating and this may provide paternity assurance for the first mate. A spermatheca that is elastic and expands to accommodate successive ejaculates potentially enables the female function to store and to use sperm of more than one mating partner, which could enhance the chance of cryptic mate choice (Simmons, 2001). However, this hypothesis is difficult to test, because the amount of sperm stored from each mating partner has to be assessed.

Sperm utilization in *A. arbustorum* changed to second-mate precedence when the interval between matings exceeded 300 days (Baur, 1994a), suggesting a reduced viability or a reduced resistance of "old" allosperm (Rogers & Chase, 2002). However, analyses of long-term sperm utilization revealed clear differences among individuals, ranging from first-mate sperm precedence, through sperm mixing, to last-mate sperm precedence (Baur, 1994a). A variable sperm precedence pattern might be explained by the differential use of sperm from different mates, but might also be due to differences in the amount of sperm received from each mate as well as sperm quality (Baur, 1994a). Thus, different factors may influence sperm utilization patterns in this snail species.

The transfer of large numbers of sperm should lead to a numerical superiority of these sperm in the storage organ. In *A. arbustorum*, however, the amount of sperm stored seems to be unaffected by the amount of sperm received (see also Bojat and Haase 2002). This suggests either an active role of the sperm receiver in transferring sperm into the storage organ, as has been found in insects (Bloch Qazi et al., 1998; Hellriegel & Bernasconi, 2000), or different quality (swimming speed, longevity) of sperm from different sperm donors (e.g. Garcia-Gonzalez and Simmons, 2005; Minoretti and Baur, 2006). We found no indication of a fixed proportion of sperm being digested and this also might be related to flexible adjustments of sperm use by the sperm receiver. The low amount of sperm reaching the storage organ of *A. arbustorum* is comparable to that in other snail species [0.1% in *H. pomatia*, Lind (1973), and 0.025% in *H. aspersa*, Rogers and Chase (2001)].

In many animal taxa, the spermatheca coevolves with sperm or ejaculate traits, possibly due to a conflict between the sex functions over the control of paternity. This indicates that the storage organ allows the sperm receiver at least some control over sperm use and fertilization (Birkhead & Pizzari, 2002). Consistent with this idea, there are indications for a coevolution between spermatheca volume and the number of sperm transferred in *A. arbustorum* (Beese et al., 2006). Thus, postcopulatory sexual selection seems to drive evolutionary changes in reproductive trait morphology and may therefore also be the reason for the complexity and diversity of sperm storage organs in helicid snails.

The type of spermatheca found in *A. arbustorum* allows a spatial separation of sperm from different mating partners. However, sperm uptake, storage and use might be influenced by both the sperm donor and the sperm receiver in different ways. This makes it difficult to determine single effects on paternity success. Nevertheless, the identification of

different mechanisms underlying sperm transfer and sperm storage is an important step to explain the observed patterns of sperm precedence.

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CHAPTER IV

BURSA TRACT DIVERTICULUM IN THE HERMAPHRODITIC LAND SNAIL *ARIANTA ARBUSTORUM* (STYLOMMATOPHORA: HELICIDAE): MORPHOLOGY, FUNCTION, AND EVOLUTIONARY IMPLICATIONS

Kathleen Beese, Konstantin Beier & Bruno Baur

ABSTRACT

A bursa tract diverticulum is widespread in the female part of the hermaphroditic reproductive system of stylommatophoran pulmonates. However, the ultrastructure of the diverticulum is unknown and there is only anecdotal evidence for a spermatophore-dissolving function for this organ. In the present study, we examined the ultrastructure of the diverticulum and investigated histological, histochemical and morphometric changes at different time intervals after mating in the simultaneously hermaphroditic land snail *Arianta arbustorum*. The diverticulum in this species of snail is a prominent organ, consisting of a luminal columnar epithelium surrounded by a thick layer of connective tissue. During mating, the diverticulum functions as the site of spermatophore uptake. Within the lumen of the diverticulum the spermatophore wall is dissolved or at least partly broken down. The digested material is taken up by epithelial cells and accumulated in molluscan-specific cells of the connective tissue, the so-called rhogocytes. Subsequent to copulation, the total diameter of the diverticulum increases markedly, reaching a maximum size 12 hours after mating, while at the same time the thicknesses of the diverticulum wall and diverticulum epithelium decrease. The length of the diverticulum shows a positive allometry and a high phenotypic variation compared to snail size, which suggests that the diverticulum is under directional sexual selection. We propose that the diverticulum in *A. arbustorum* has evolved in response to selection pressures imposed by divergent evolutionary interests between male and female function.

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INTRODUCTION

The reproductive system of stylommatophoran pulmonates, a highly diverse hermaphroditic gastropod group, possesses a sacculate reservoir adjacent to the pericardium. This so-called bursa copulatrix (=gametolytic gland; Tompa, 1984) is commonly connected to the female reproductive system via a thin duct. As a lateral continuation of the lower part of the bursa duct, the bursa tract diverticulum is widespread in the Stylommatophora and apparently plesiomorphic (Barker, 2001; Koene & Schulenburg, 2005). The diverticulum is a blind-ended tube, which is especially long in several species, e.g., in *Eobania vermiculata* (Tompa, 1984). In *Helix pomatia*, the length of the diverticulum is highly variable; in some individuals it may also be reduced or entirely lacking (Hochpoechler & Kothbauer, 1979; van Osselaer & Tursch, 2000). The origin of the diverticulum is not clear. It has been suggested that the separation of the allospermiduct led to the evolution of the diverticulum (Hochpoechler & Kothbauer, 1979; Visser, 1981). However, there is no convincing evidence for this hypothesis.

In many pulmonates, spermatozoa and seminal fluids are transferred to the mating partner by means of a spermatophore (Tompa, 1984). The spermatophore is formed in the epiphallus and flagellum, and is largely composed of secretory material (containing glycosaminoglycans and mucoproteins; Mann, 1984). The spermatophore shows a species-specific morphology with taxonomic significance (Baur, 1998). The bursa tract diverticulum, when present, is specifically positioned relative to the bursa duct opening to function as the site of spermatophore receipt from the mating partner (Barker, 2001). Sperm received (allosperm) travel up the spermoviduct to reach the tubules of the spermatheca, where they are stored until fertilization (Lind, 1973). The vast majority of allosperm (99.98% in *Helix aspersa*; Rogers & Chase, 2001), however, is transferred into the sacculate reservoir of the bursa copulatrix (Lind, 1973). The bursa was originally thought to function as the site of allosperm storage (Meisenheimer, 1912) or sperm activation (cf. Rogers & Reeder, 1987). However, ultrastructural and histochemical studies suggested that its function may be the extracellular digestion and subsequent resorption of excess gametes (primarily allosperm) and other reproductive products, such as secretions from the albumen gland, oviductal glands, and seminal channel (Németh & Kovács, 1972; Rogers et al., 1980; Gomez et al., 1991).

The morphology and function of the bursa copulatrix are relatively well known (e.g., Rogers et al., 1980; Kitajima & Paraense, 1983; Rogers & Reeder, 1987). The fine structure of the diverticulum, however, has not yet been studied, although the widespread occurrence of this organ strongly suggests an adaptive function. Several studies indicate a spermatophore-dissolving function. For example, in *Helix aspersa* the spermatophore begins to dissolve 68 h after copulation (Adamo & Chase, 1988) and in individuals of *Mastus olivaceus* collected in the field, one to three partly dissolved spermatophores were found in the distal part of the diverticulum (Parmakelis & Mylonas, 2002). Light microscopic investigations carried out in *Arianta arbustorum* (Eichardt, 1949) and *Theba pisana* (Noyce, 1973) support this hypothesis. Furthermore, Hofmann (1923) assumed that in *A. arbustorum* spermatozoa are only able to migrate to the storage organ after the spermatophore capsule has been dissolved in the diverticulum.

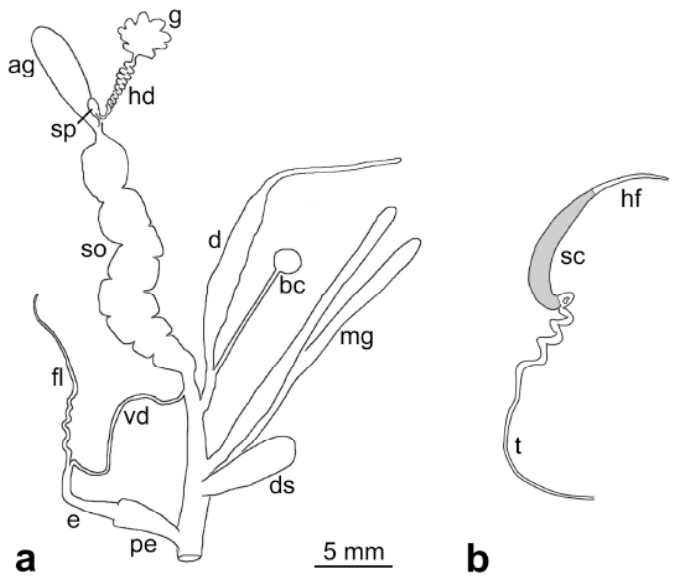

Figure 1. Schematic representation of (**a**) the reproductive tract and (**b**) the spermatophore of *Arianta arbustorum*, both drawn on the same scale. ag, albumen gland; bc, bursa copulatrix; d, diverticulum; ds, dart sac; e, epiphallus; hf, head filament; fl, flagellum; g, gonad; hd, hermaphroditic duct; mg, mucous glands; pe, penis; sc, sperm container; so, spermoviduct; sp, spermatheca; t, tail; vd, vas deferens.

The simultaneously hermaphroditic land snail *Arianta arbustorum* has been extensively studied with respect to mating behavior and reproductive morphology (Baur, 1988; Haase & Baur, 1995; Baminger & Haase, 2000; Bojat & Haase, 2002) (Fig. 1a). Because individuals mate repeatedly during a reproductive season and store viable allosperm for more than one year, the mating system and reproductive tract are potentially subject to different mechanisms of sexual selection (sexual conflict, sperm competition, or cryptic female choice). Mating in *A. arbustorum* lasts several hours and includes elaborate courtship behavior with optional dart shooting. Copulation is reciprocal and after simultaneous penis intromission each snail transfers one spermatophore into the partner's

diverticulum. The spermatophore has a distinctive form consisting of head filament, a 0.6–1 cm long sperm container, and a 2–3 cm long tail whose proximal part is spirally coiled (Hofmann, 1923) (Fig. 1b). With about 2.2 cm in length, the diverticulum is ~1.5 times as long as the bursa copulatrix and is thus a prominent organ in this species.

In the present study, we examined the ultrastructure of the bursa tract diverticulum in *A. arbustorum*. To reveal the adaptive function of the diverticulum, we analyzed structural changes of this organ after mating using morphometric, histological, and histochemical methods. Furthermore, we assessed the duration of spermatophore presence and sperm survival in the spermatophore. We evaluated the interindividual variation in diverticulum length in relation to shell size because traits under directional sexual selection often show positive allometry (cf. Lüpold et al., 2004) and exhibit a high degree of phenotypic variation (Pomiankowski & Møller, 1995). We also propose a hypothesis for the evolution of spermatophore-receiving structures in helicid snails.

MATERIALS AND METHODS

Animals

Subadult specimens of *Arianta arbustorum* (Linnaeus, 1758) were collected near Nuglar in northern Switzerland (47°29'N, 7°42'E; 430 m above sea level) in spring 2004. They were isolated upon arrival in transparent plastic beakers (6.5 cm in diameter, 8 cm deep) and maintained under a reversed 16-h light : 8-h dark photoperiod at temperatures of 19° : 17°C. Sexual maturation, as indicated by the formation of a reflected shell lip, required 8 weeks in the laboratory. During this time the snails were kept on soil from their natural habitat and fed fresh lettuce *ad libitum*. Mating trials with 70 virgin individuals were set up in a large transparent plastic box (34 × 22 × 9.5 cm) in which snails were allowed to court freely. After they had copulated, snails were randomly assigned to one of 10 groups with three snails per group. The diverticulae of the snails were examined 6 h, 12 h, 1 day, 2, 3, 4, 5, 7, 10, and 22 days after mating.

Histology and morphometry

All mated and three virgin snails were killed by decapitation and dissected. Cylindrical sections of the middle region of the diverticulum were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) and subsequently embedded in paraplast for light microscopic investigations (LM). Cross-sections 5–7 μm thick were stained with

haematoxylin-eosin or azan. Histochemical characterization included the staining with PAS for polysaccharides (including glycogen), Alcian blue 8GX, pH 2.5, for acid glycosaminoglycans (primarily carboxylated glycosaminoglycans) and Feulgen for DNA (Romeis, 1989). For transmission electron microscopy (TEM), small pieces of the diverticulum wall were immediately fixed in 3% paraformaldehyde and 0.5% glutaraldehyde in 10 mM PBS, pH 7.4, according to Karnovsky (1965). The samples were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 h and rinsed several times in 0.1 M PBS and water. Following dehydration, including en bloc staining in 70% ethanol + 2% uranyl acetate, specimens were embedded in Epon. Ultrathin sections (70 μm) were made on a Reichert-Jung Ultracut, collected on formvar-coated grids, stained with 6% uranyl acetate for 1 h followed by lead acetate for 2 min, and examined with a Zeiss EM 910 TEM.

Measurements of total diverticulum diameter at its broadest part were obtained prior to fixation using digitized images taken during dissections with a Sony CCD-Iris camera mounted on a Leica MZ 8 binocular microscope and compared to the size in virgin snails. Digitized microscopic images of paraplast-embedded, cross-sectioned diverticulum mid-pieces served to estimate the thicknesses of the diverticulum wall and epithelium. Distances were measured using NIH Image Version 1.63 (http://rsb.info.nih.gov/nih-image). Mean values of five measurements were used in the analyses.

The presence/absence of a spermatophore or spermatophore remnants in the diverticulum was recorded. When a spermatophore was present its content was placed in a drop of Ringer solution (Romeis, 1989) on a microscope slide and checked for motile spermatozoa.

Variation in length of the diverticulum

A sample of 22 virgin snails was used to estimate the interindividual variation in diverticulum length. Prior to dissection, we measured the shell width of each snail to the nearest 0.1 mm using a vernier caliper. The diverticulum was fixed in 70% ethanol and pinned out straight. Using digitized images, we measured the length from the point where the bursa stalk branches off to the uppermost tip of the diverticulum. Measuring soft parts can be problematic because there are several potential sources of artificial variance (Jordaens et al., 2002). Seasonal (Emberton, 1985; Armbruster, 1994) and developmental (Bride & Gomot, 1991) variation in diverticulum length can be excluded because we used snails of the same age. Furthermore, fixation should have the same effects on all

individuals (Meier-Brook, 1976). To increase precision and repeatability of measurements, the pinning out of the diverticulum was repeated five times and each image was measured five times. The repeatability of measurements was 0.99, indicating a high accuracy (Lessells & Boag, 1987). The interindividual variation was evaluated by calculating the coefficient of variation (CV = standard deviation × 100 / mean) (Sokal & Rohlf, 1995).

RESULTS

Histology of the diverticulum

The bursa tract diverticulum of *Arianta arbustorum* is round to ovoid in cross-section and consists of an inner epithelial lining surrounded by a pronounced connective tissue layer. Basal to the epithelium there is a 3D network of muscles in the connective tissue.

The slightly folded, single-layered, columnar epithelium is composed of one cell type with oval-shaped or elongated nuclei that are situated in the basal thirds of the cells (Fig. 2a). The luminal cell membrane is folded into a dense, microvillous brush border (Fig. 2a,b). Cytoplasmic inclusions of different sizes with granular contents and glycogen particles lie apically (Fig. 2a). These cytoplasmic inclusions stain PAS-positive, indicating the presence of polysaccharides (including glycogen) in this area (Fig. 8a). The apical cytoplasm of the epithelial cells also stains intensely red with azan. Most of the mitochondria, which are elongated and have longitudinal cristae, are found in the apical part of the cells (Fig. 2b,c). In addition, this region is rich in vesicles with different contents (Fig. 2a,b). Basal to zonulae adhaerentes and septate desmosomes, cells adhere to each other through interdigitations, which extend to the bases of the cells (Fig. 2a,d). Golgi complexes (Fig. 2d) and elements of endoplasmic reticulum are found in proximity to the cell nucleus. The epithelium of the basal region of the cell is characterized by deep, finger-like invaginations (Fig. 2a,e). Cytoplasmic inclusions with glycogen and granular contents are also located in this region. Beneath the basal lamina, the connective tissue layer of the diverticulum wall consists of a network of collagen and/or reticular fibrils embedding smooth muscle-cells (Fig. 2e). The sarcoplasmic reticulum of the muscle cells exhibits several cisterns and some of the muscle cells enclose large glycogen deposits.

The most characteristic elements of the connective tissue are numerous vesicular cells of irregular shape that have an unusual cell surface structure (Fig. 3a). These cells are also found in the connective tissue of other snail species and have been depicted as rhogocytes (=pore cells, Kugelzellen, Blasenzellen, globular cells; Haszprunar, 1996). The

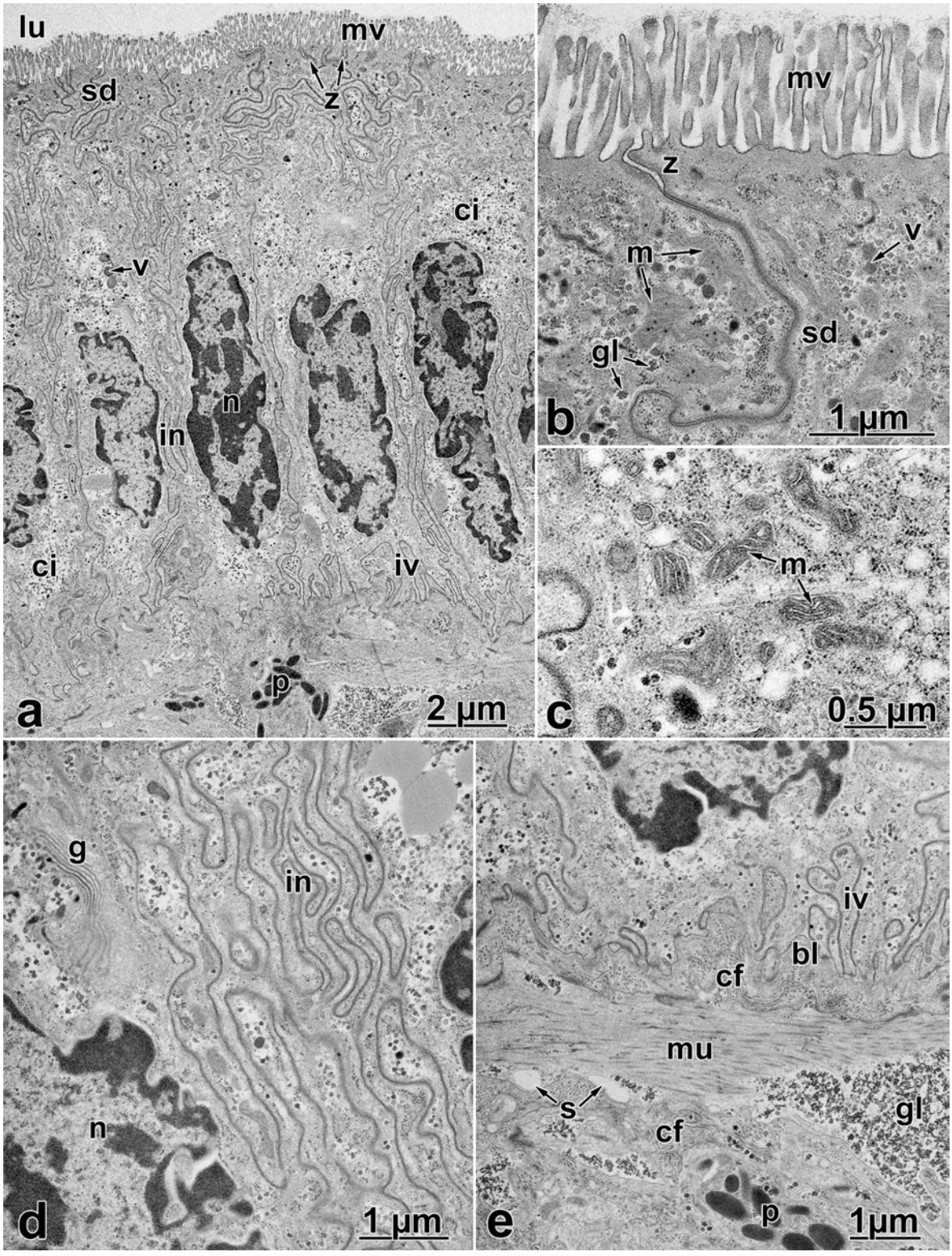

Figure 2. *Arianta arbustorum*. Epithelium of the diverticulum of a virgin snail. TEM. **a:** Low-power micrograph of epithelium. **b:** Apical region of epithelial cells. **c:** Mitochondria in apical region of cells. **d:** Lateral cell membrane. **e:** Basal region of epithelial cells; muscle layer and pigment cells beneath epithelium. bl, basal lamina; cf, connective tissue fibrils; ci, cytoplasmic inclusions; g, Golgi complex; gl, glycogen; in, interdigitating plasma membranes; iv, invaginations; lu, lumen of diverticulum; m, mitochondria; mu, muscle cell; mv, microvilli; n, nucleus; p, pigment cell; s, sarcolemma with cisterns; sd, septate desmosome; v, vesicles; z, zonula adherens.

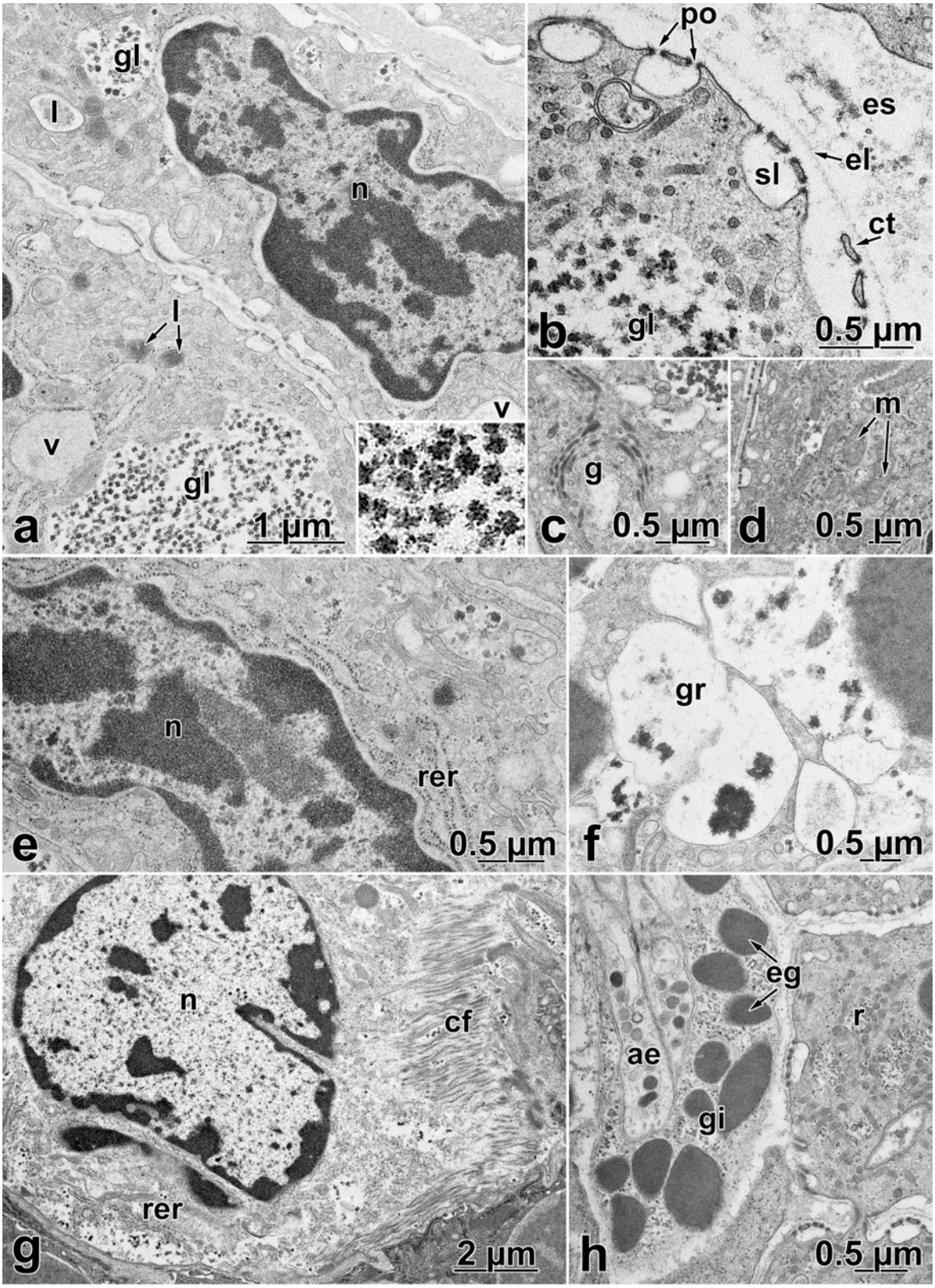

Figure 3. *Arianta arbustorum*. Connective tissue of the diverticulum of a virgin snail. TEM. **a:** Rhogocyte. Inset: α-rosettes of glycogen. **b:** Detail of cell surface of rhogocytes. **c:** Golgi apparatus. **d:** Mitochondria. **e:** Rough endoplasmic reticulum in rhogocytes. **f:** Detail of granular cell. **g:** Fibroblast-like cell. **h:** Axon ending and glial cell. ae, axon ending; cf, connective tissue fibrils; ct, cytoplasmic tongue; eg, electron-dense granules; el, external lamina; es, extracellular space; g, Golgi complex; gi, glial cell; gl, glycogen; gr, granules; l, lysosome; m, mitochondria; n, nucleus; po, pores; r, rhogocyte; rer, rough endoplasmic reticulum; sl, surface lacunae; v, vesicles.

rhogocytes are characterized by a complex system of tubular or vesicular invaginations of the cell surface bridged by cytoplasmic tongues. The invaginations communicate with the extracellular space through pores or slits, forming a system of surface lacunae termed Spaltenapparat or sieve system (Fig. 3b). An external, finely fibrillar coat (external lamina) surrounds the rhogocytes (Fig. 3b), and there are no junctional contacts to any other cell. The rhogocytes are usually very large and usually have round nuclei. Numerous lysosomes, small vesicles and granules are scattered throughout the cytoplasm. A strongly developed Golgi apparatus (Fig. 3c) and a high number of mitochondria (Fig. 3d) are found in the rhogocytes. A prominent part of the cell is formed by the rough endoplasmic reticulum (RER), which is frequently located near the nucleus (Fig. 3e). The cisterns of the RER contain fine granules. The cytoplasm stains only faintly with azan, but some of the rhogocytes are PAS positive (Fig. 8a) and, as revealed by TEM, contain large vacuoles with glycogen granules in the form of β-particles and α-rosettes (inset in Fig. 3a).

Other types of connective tissue cells include granular cells filled with a large number of electron-bright granules (Figs. 3f, 6a), fibroblasts showing collagen fibrils in the cytoplasm (Fig. 3g), and spindle-shaped pigment cells that contain many pigment granules (Figs. 2e, 7c). In addition, in close relationship to the muscle layer below the diverticulum epithelium, glial cells (characterized by large vesicles with electron-dense contents) accompany axon endings (Fig. 3h). The axon endings contain mainly light- and dark-cored vesicles, clear vesicles, and vesicles with granular content.

Mating effects

Morphometry. Due to a mechanical stretching caused by the uptake of a spermatophore, the total diameter of the diverticulum increases during the first 6 hours after mating (Fig. 4a). Surprisingly, the increase proceeds markedly to a maximum size 12 hours after mating. This is followed by a continuous decline, and 22 days after mating the total diverticulum diameter is similar to that in virgin snails. The opposite effects are found for the thickness of the diverticulum wall (Fig. 4b) and the thickness of the epithelium (Fig. 4c). Uptake of the spermatophore results in a decrease of the thickness of the diverticulum wall over 12 hours. The thickness of the diverticulum epithelium reaches a minimum size 2 days after mating. Both the thicknesses of the diverticulum wall and of the epithelium return to the initial size after 22 days.

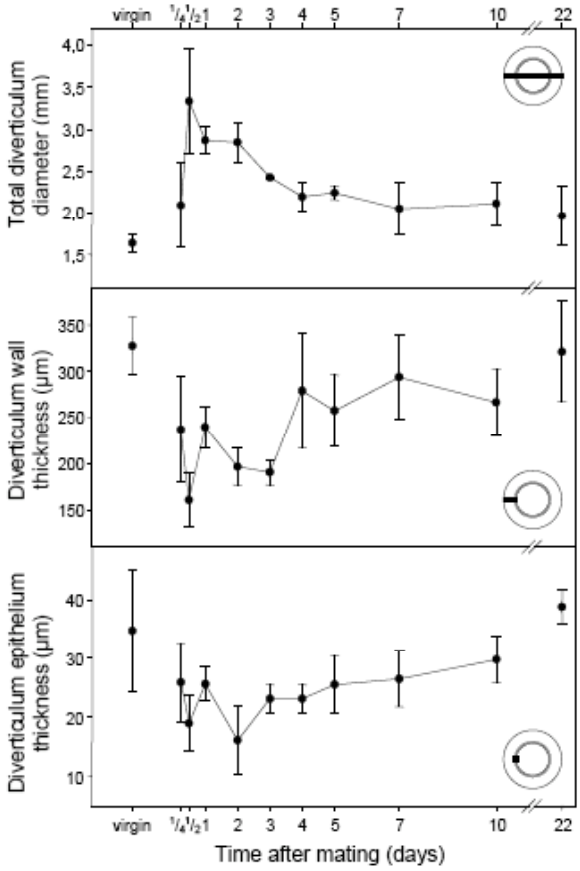

Figure 4. Morphometry of the diverticulum in virgin snails and in snails after mating (6 h, 12 h, 1 day, 2, 3, 4, 5, 7, 10 and 22 days). **a:** Total diverticulum diameter. **b:** Diverticulum wall thickness. **c:** Diverticulum epithelium thickness. Means ± SD.

Diverticulum histology: postmating. Six hours after mating, the histology and histochemistry of the diverticulum wall differ from that of virgin snails. In the epithelial cells there is a significant increase in the number and sizes of PAS-positive cytoplasmic inclusions, both apically and basally of the cell nuclei (Figs. 5a, inset in 8b). In addition to glycogen and granules, myelin-like figures are found within the basally located cytoplasmic inclusions (Fig. 5a). At the bases of some microvilli signs of endocytotic-like activity can be seen (Fig. 5b) and between the lateral membranes intercellular spaces are opening (Fig. 5c). The invaginations of the basal lamina are reduced compared to the epithelium in virgin snails (Fig. 5a).

At the light microscopic level, 6 hours after mating the connective tissue of the diverticulum wall stains intensely PAS-positive (Fig. 8b) and light blue with azan (Fig. 9a). Corresponding TEM sections reveal vast numbers of small- and medium-sized, electron-lucent as well as electron-dense vesicles within the rhogocytes (Fig. 6a). Enlarging, electron-lucent, fluid-filled vacuoles (Fig. 6a), numerous vesicles with inclusions of different electron densities (Fig. 6b), and small spherical vesicles appear in the cytoplasm of this cell type. The surface lacunae of the sieve system show endocytotic-like formation of vesicles at their bases (Fig. 6c).

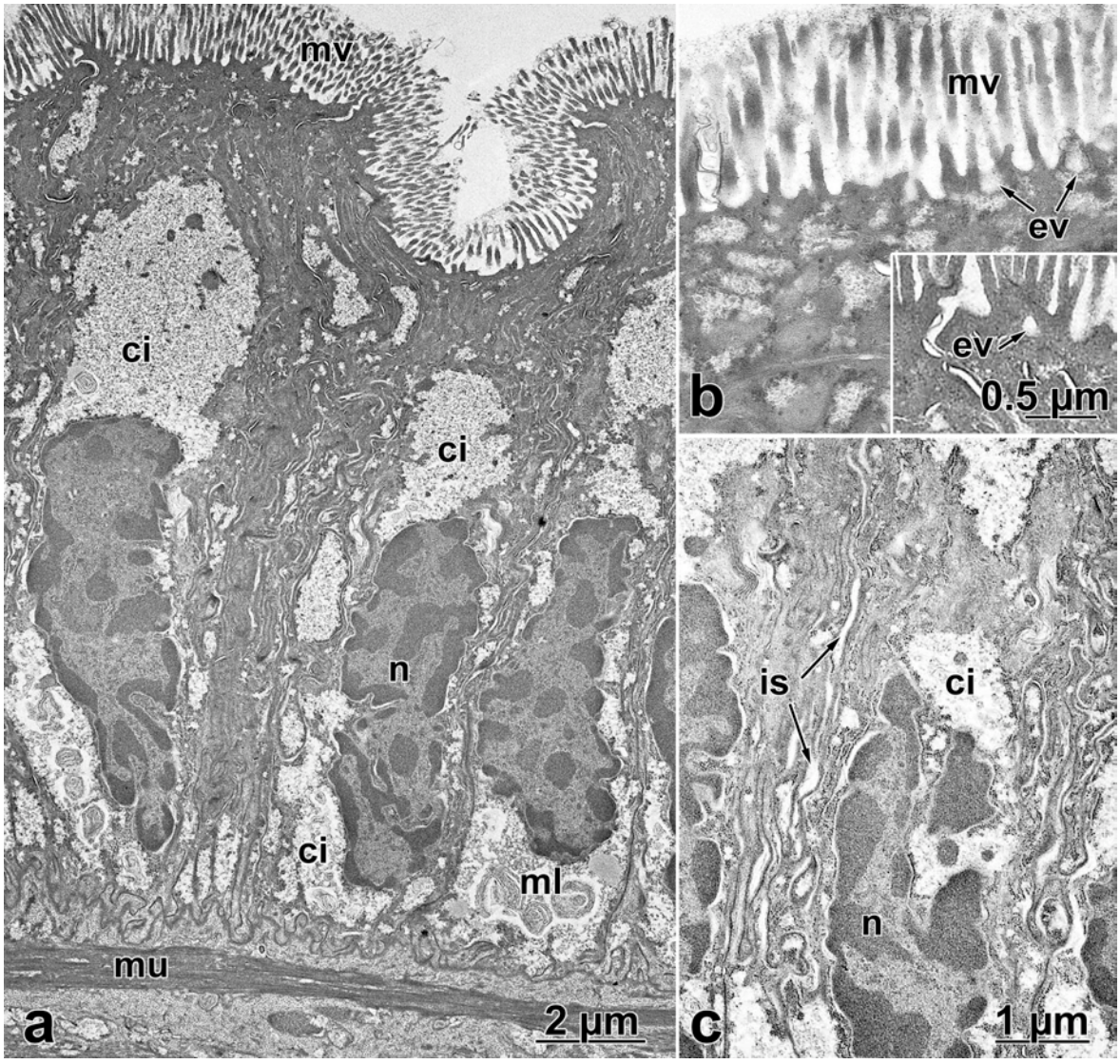

Figure 5. *Arianta arbustorum*. Epithelium of the diverticulum. Six hours postmating. TEM. **a:** Low-power micrograph of the epithelium. **b:** Apical region of epithelial cells showing the formation of endocytotic vesicles at the base of some microvilli. **c:** Lateral cell membranes of epithelial cells with intercellular spaces. ci, cytoplasmic inclusions; ev, endocytotic vesicles; is, intercellular spaces; ml, myelin-like figures; mu, muscle cell; mv, microvilli; n, nucleus.

During the following days after mating, the diverticulum epithelium is stretched and the cells show a more cuboidal shape (Fig. 7a). This is in agreement with the morphometric results. The cell nuclei are arranged diagonally and the basal lamina possesses no finger-like invaginations (Fig. 7a,c). The apical cell surface shows less endocytotic-like activity compared with that of animals 6 hours after mating (Fig. 7b). The number and size of PAS-positive areas within the epithelial cells decrease, but the TEM sections reveal several large vesicles within and beneath the epithelium (Fig. 7a,c).

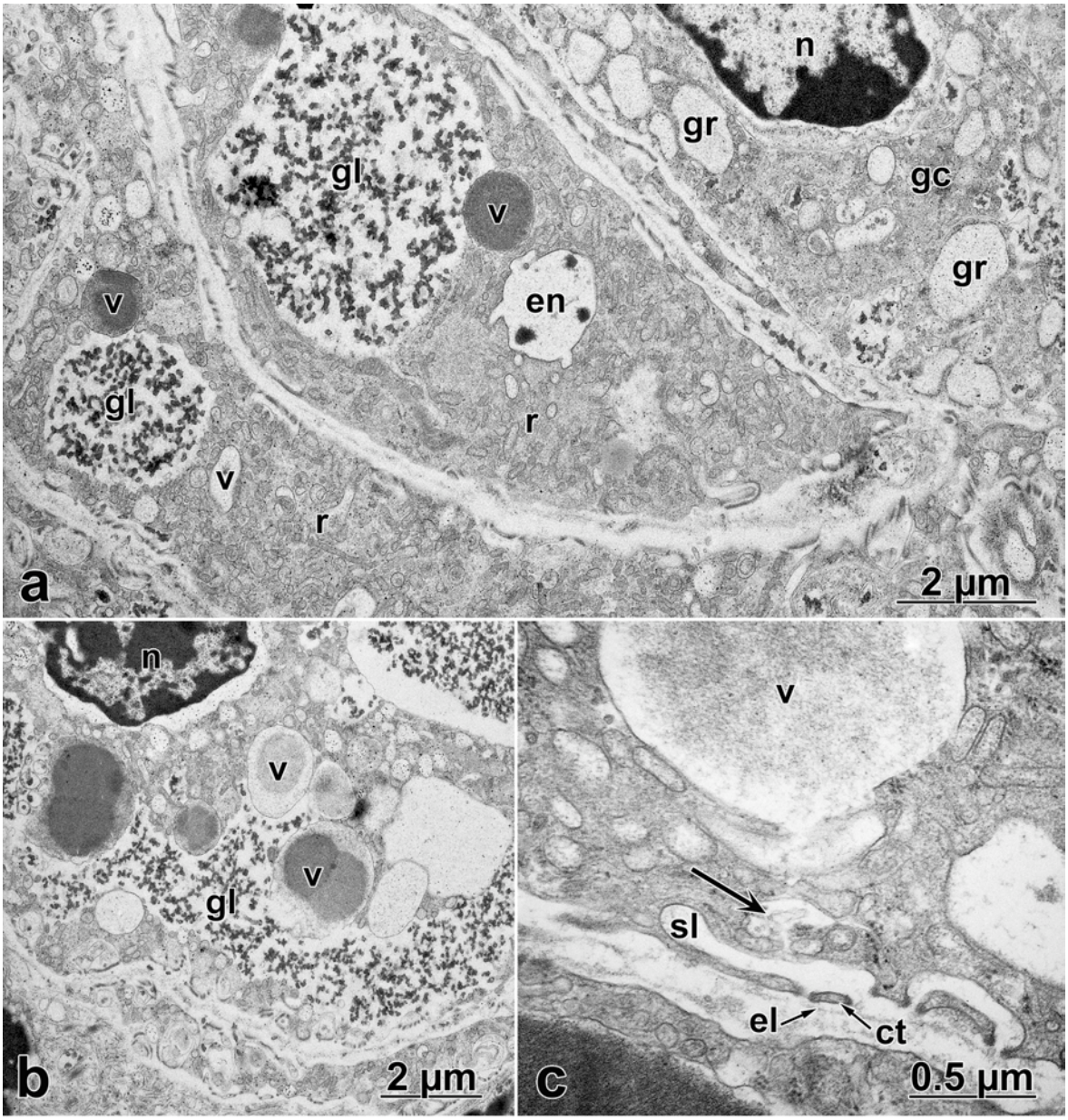

Figure 6. *Arianta arbustorum*. Connective tissue of the diverticulum. Six hours postmating. TEM. **a:** Low-power micrograph of connective tissue with rhogocytes and granular cell. **b:** Detail of rhogocyte with vesicles and glycogen deposits. **c:** Detail of the Spaltenapparat of a rhogocyte with endocytotic-like formation of vesicles (large arrow). en, enlarging vesicle; ct, cytoplasmic tongue; el, external lamina; gc, granular cell; gl, glycogen; gr, granules; n, nucleus; r, rhogocytes; sl, surface lacunae; v, vesicles.

Within the rhogocytes, the size and number of electron-dense vesicles increase until around day 4 after mating (probably via fusion of smaller vesicles), so that even the cell nuclei are deformed and displaced from their central positions (Fig. 7d). The PAS- and azan-stained sections show an intensity gradient, declining from the luminal region of the connective tissue to the outside (Fig. 10a,b). From day 4 on after mating, the sizes of the vesicles within the rhogocytes begin to decline. At day 22 small vesicles remain, accompanied by large glycogen deposits (Fig. 7e).

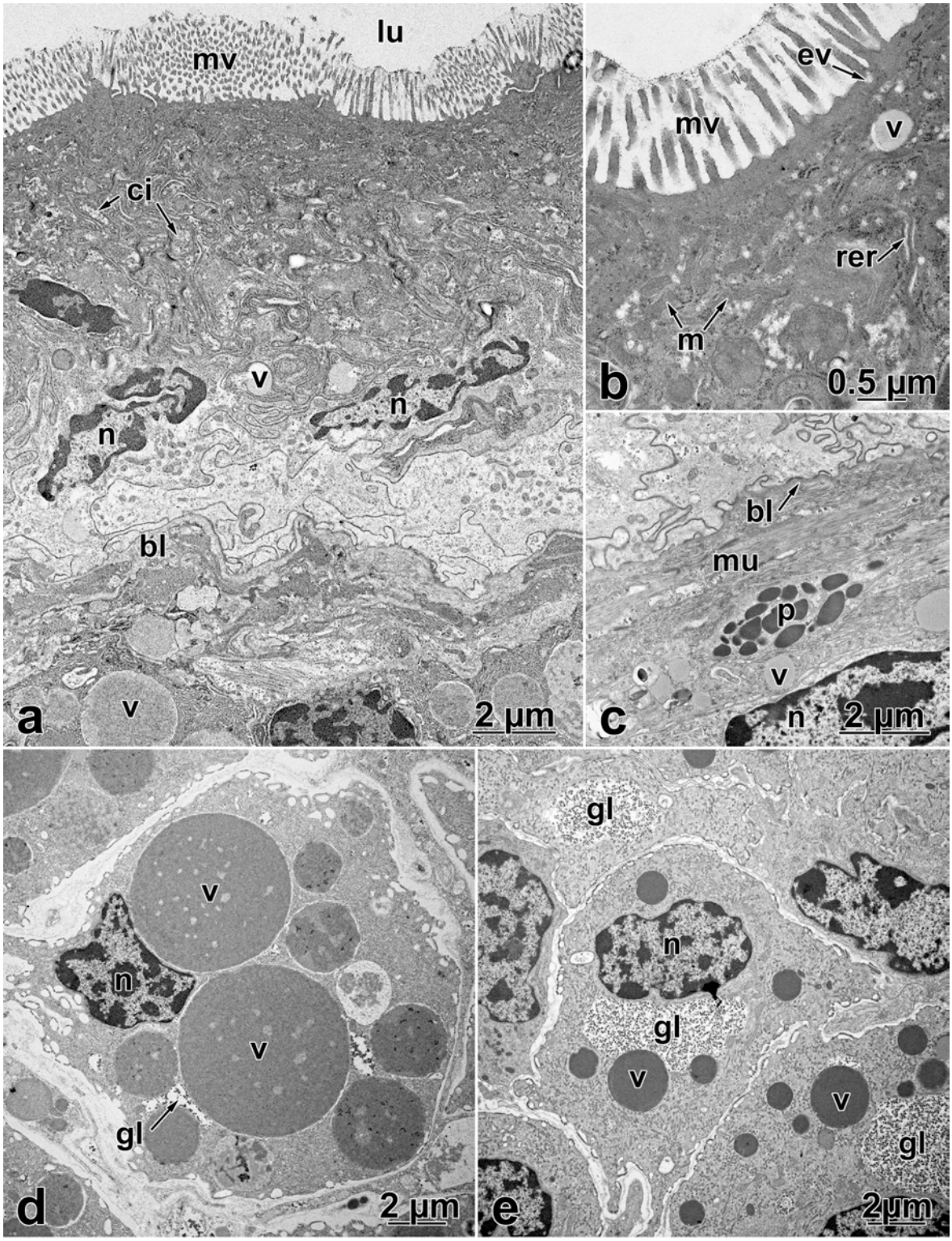

Figure 7. *Arianta arbustorum*. Epithelium and connective tissue of the diverticulum. **a,b**: Two days postmating, **c,e**: Twenty-two days postmating. **d**: Four days postmating. TEM. **a:** Low-power micrograph of epithelium. **b:** Apical region of epithelial cells. **c:** Layer beneath epithelium. **d,e:** Rhogocytes. bl, basal lamina; ci, cytoplasmic inclusions; gl, glycogen; lu, lumen of diverticulum; m, mitochondria; mu, muscle cell; mv, microvilli; n, nucleus; p, pigment cell; rer, rough endoplasmic reticulum; v, vesicles.

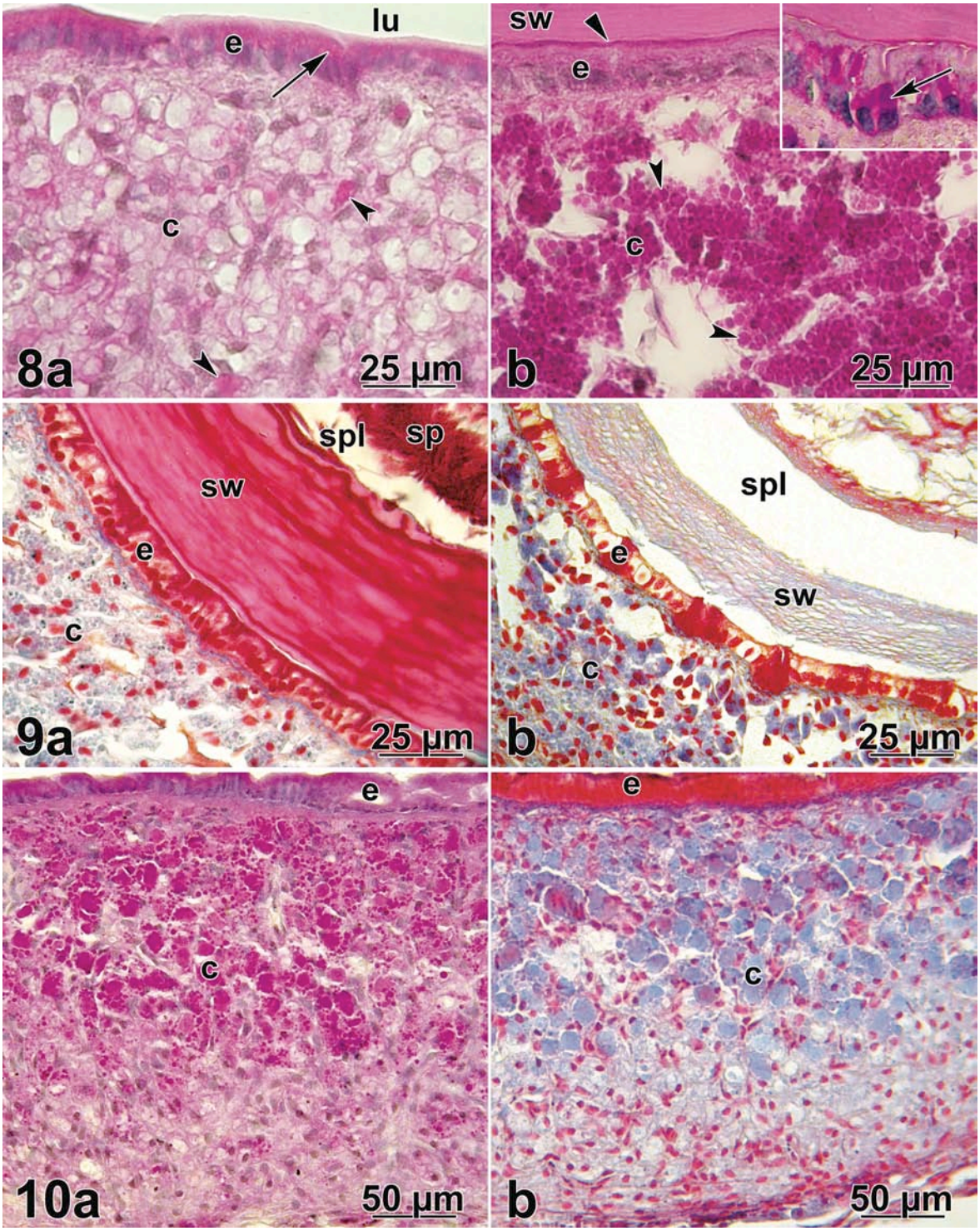

Figure 8. *Arianta arbustorum*. Diverticulum wall. LM. PAS. **a:** Virgin snail. **b:** Six hours postmating. PAS-positive staining (pink-magenta colored) of epithelial cells (arrows), rhogocytes (indented arrowheads) and lumen between epithelium and spermatophore (arrowhead). c, connective tissue; e, epithelium; lu, lumen of diverticulum; sw, spermatophore wall.

Figure 9. *Arianta arbustorum*. Diverticulum containing a spermatophore. LM. Azan. **a:** Six hours postmating. **b:** Five days postmating. c, connective tissue; e, epithelium; sp, spermatozoa; spl, spermatophore lumen; sw, spermatophore wall.

Figure 10. *Arianta arbustorum*. Diverticulum wall 4 days postmating showing an intensity gradient of PAS- and azan-staining cells. LM. **a:** PAS. **b:** Azan. c, connective tissue; e, epithelium.

Spermatophore dissolution. In the time course after mating, the spermatophores disappear from the diverticulum (Fig. 11a–e). Up to 4 days after mating, spermatophores are found in the diverticulae of all examined individuals. On day 7, in two of three dissected snails the diverticulae contain spermatophores, and on day 5, 10 and 22 in only one of three snails is a spermatophore found. On day 10, two individuals show remnants of spermatophores located in the stalk of the bursa copulatrix. The number of spermatozoa encapsulated in the spermatophore apparently decreases following day 3 after mating (Fig. 11f–j) and active spermatozoa are found until 5 days after mating. After 7 days, just a few spermatozoa are still alive, and 10 and 22 days after mating only immotile remnants of sperm cells are found. Some of these spermatozoa still have intact sperm heads, as indicated by Feulgen staining.

With increasing time intervals after mating, the structure of the spermatophore wall within the diverticulum changes. Light microscopy reveals a compact spermatophore wall 6 hours after mating, which stains intensely red with azan (Figs. 9a, 11a). The spermatozoan tails are PAS-positive and the narrow lumen between epithelium and spermatophore stains Alcian blue- and PAS-positive (Fig. 8b). Three days after mating the spermatophore wall starts to become porous (Fig. 11c) and 5 days after mating only a small red stripe remains close to the lumen of the spermatophore wall, while the rest stains light blue with azan (Figs. 9b, 11d).

Diverticulum length variation

There is a positive allometric relationship between diverticulum length and shell width of the snail (linear regression; $r = 0.62$, degrees of freedom (df) 20, $P < 0.01$). The interindividual coefficient of variation (CV) of diverticulum length (CV = 10.91%) is more than two times greater than that of shell width (CV = 4.09%).

DISCUSSION

Histology and function of the diverticulum

The histological and histochemical evidence presented here indicates that the diverticulum in *Arianta arbustorum* digests or at least partly breaks down received spermatophores. This confirms the hypothesis of a spermatophore-dissolving function of this organ in *A. arbustorum* and other species of stylommatophoran snails (Hofmann, 1923; Eichardt, 1949; Noyce, 1973; Adamo & Chase, 1988; Parmakelis & Mylonas, 2002).

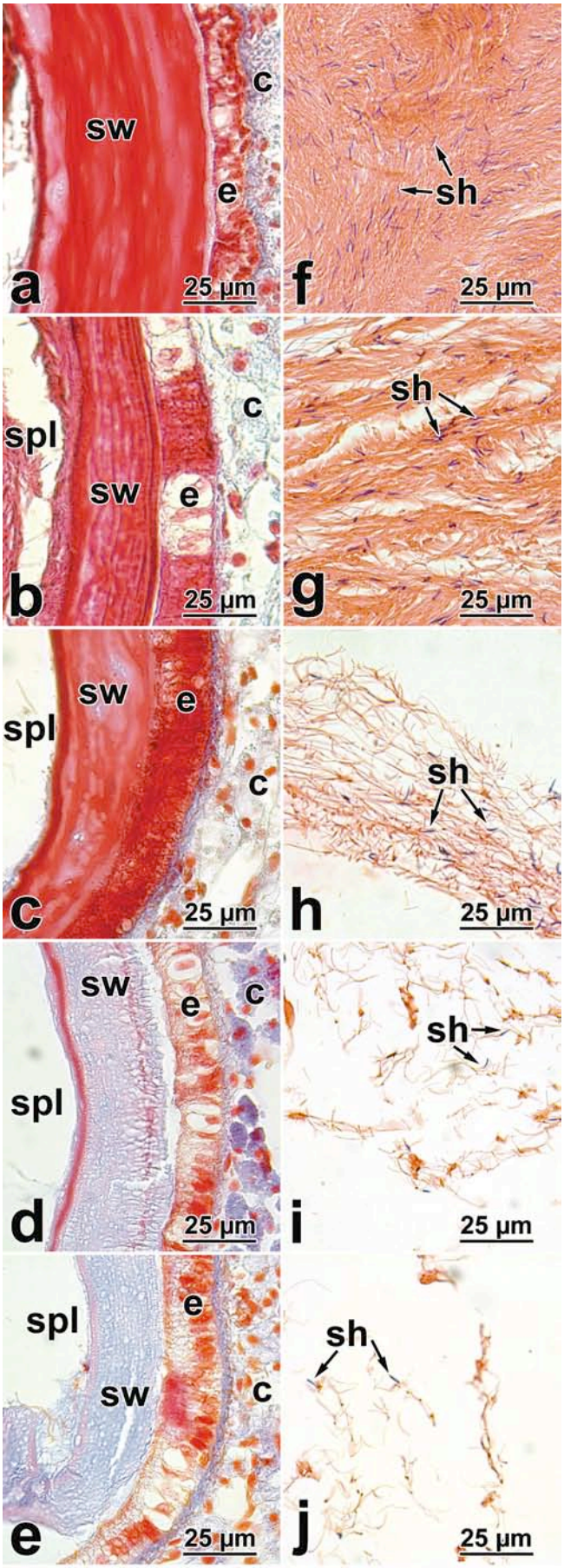

Figure 11. *Arianta arbustorum*. Spermatophore dissolution. **a–e**: Structural changes of the spermatophore wall. LM. Azan. **f–j**: Decrease of sperm number in spermatophore. LM. Hematoxylin-eosin. **a,f:** Six hours postmating. **b,g:** One day postmating. **c,h:** Three days postmating. **d,i:** Five days postmating. **e, j:** Ten days postmating. c, connective tissue; e, epithelium; sh, sperm heads; spl, spermatophore lumen; sw, spermatophore wall.

The spermatophore substance seems to be dissolved by fluids containing digestive enzymes that are released into the lumen shortly after mating (Hofmann, 1923; Eichardt, 1949). In the present study, we found an increase in total diverticulum diameter 6–12 h after mating, while at the same time the thickness of the diverticulum wall decreased. There was also a decrease in the thickness of the diverticulum epithelium until 2 days after mating. These size changes can be attributed to fluid secretions (Hofmann, 1923). Furthermore, although we could not observe secretory vesicles in the epithelial cells, enzyme secretion must have occurred at some point because within a few days after mating the spermatophore wall changed from a compact to a porous structure. The presence of acidic glycosaminoglycans (components of the spermatophore wall; Mann, 1984) and carbohydrates in the luminal layer between epithelium and spermatophore wall further suggest the release of digestive enzymes. Moreover, similar to the epithelium of the mammalian small intestine, the ultrastructure of the diverticulum epithelium—including a microvillar brushborder, apical cellular junctions, labyrinthine plasma membranes, and typical organelle distribution—indicates an absorptive function. Shortly after the snails mated there was an increase in the size of PAS-positive areas in the epithelial cells and intercellular spaces opening between the lateral cell membranes occured. Both processes indicate an uptake of substances from the lumen.

Subsequently, as suggested by ultrastructural observations, substances received with the spermatophore seem to be endocytosed by the rhogocytes of the diverticulum wall, temporarily incorporated, and processed during the days following copulation. This is indicated by the occurrence of large vesicles within the rhogocytes shortly after mating and their subsequent size decrease. Rhogocytes are a specific molluscan type of connective tissue cell that surrounds many organs. Several functions have been attributed to these cells, e.g., hemocyanin and/or hemoglobin formation, collagen synthesis, accumulation of calcium, and transport and storage of nutrients or phagocytosis (Pan, 1958; Sminia, 1972; Skelding & Newell, 1975; Haszprunar, 1996; Luchtel & Deyrup-Olsen, 2001). In the rhogocytes of the diverticulum of *A. arbustorum*, we found neither hemocyanin molecules inside the surface lacunae, nor evidence for collagen synthesis or calcium accumulation. Instead, we found evidence for an endocytotic function and potentially also a carbohydrate storage function, which is indicated by the accumulation of glycogen after mating. Because molluscs have no equivalent to a glycogen-storing organ such as the vertebrate liver, or a lipid-storing tissue such as adipose tissue of mammals or fat bodies of vertebrates

(Brandriff & Beeman, 1973), the connective tissue of the diverticulum wall may function as a temporary storage area.

The efficiency of dissolving the spermatophore wall and/or the size and quality of the spermatophores transferred seem to differ markedly among individual snails. Some snails had no spermatophore left in the diverticulum 5 days after mating, while in one snail remnants of a spermatophore were still present 22 days postmating. Moreover, in two individuals spermatophore remnants were found inside the bursa copulatrix stalk 10 days after copulation. This observation supports the hypothesis that spermatophores of *A. arbustorum* are not completely dissolved in the diverticulum, but are transferred into the bursa for final digestion (Eichardt, 1949; Haase & Baur, 1995). In addition, because a previous study showed that most spermatophores had been largely or entirely transferred into the bursa copulatrix 5 days postmating (Haase & Baur, 1995), it was suggested that spermatozoa encapsulated close to the head filament of the spermatophore may have a low probability to leave it (Bojat & Haase, 2002). This result is in contrast with our study, in which most of the spermatozoa had left the spermatophore by 4 days after mating. However, the results of our study support the hypothesis that spermatozoa leave the spermatophore actively (Bojat & Haase, 2002).

Our results also indicate that the diverticulum and the bursa copulatrix have similar functions. Histochemical studies in *Helix pomatia*, *Biomphalaria glabrata*, and *Arion subfuscus* showed that the epithelial cells of the bursa release hydrolytic enzymes (acid phosphatase, DNAse, proteinase) into the lumen for extracellular digestion (Németh & Kovács, 1972; Rogers & Reeder, 1987; Gomez et al., 1991). Subsequently, the cells of the bursa copulatrix absorb and accumulate the digested material. Lipids and glycogen have been demonstrated to serve as storage products in the bursae of *A. subfuscus*, *B. glabrata* and *Sonorella santaritana* (Rogers et al., 1980; Kitajima & Paraense, 1983; Gomez et al., 1991).

Evolutionary implications

In snails without a diverticulum, received spermatophores and sperm are solely digested in the bursa copulatrix (e.g., Gomez et al., 1991). This raises the question of why two separate digestive organs with functional similarities have evolved in the female reproductive tract of *A. arbustorum* and other snail species. Spermatophores might have evolved as a nutritional donation to the mating partner and their digestion may serve as a source of nutrients for metabolism or egg production (Walker, 1980). However, because

spermatophore transfer is reciprocal in *A. arbustorum*, there is no net benefit from receiving a spermatophore because about the same amount of nutrients have to be given away as are received. Furthermore, sperm receivers could use sperm digestion as a postcopulatory mechanism to reduce or eliminate the fertilization success of sperm from low-quality mates (Birkhead et al., 1993; Eberhard, 1996), or to store sperm from more than one mating partner because individuals receive a larger amount of allosperm at a single copulation than space is available in the spermatheca. Thus, the female function could gain fitness benefits from multiple mating, such as increased offspring survival or increased genetic quality of the offspring (Tregenza & Wedell, 1998; Jennions & Petrie, 2000). However, the mentioned hypotheses cannot explain the existence of two separate digestive organs.

There is accumulating evidence for divergent evolutionary interests between the sexes driving a correlated evolution of reproductive traits (e.g., Hosken et al., 2001). Male fitness is generally limited by the number of mates, whereas female fitness by resources. Thus, males compete to secure a large number of fertilizations, whereas females tend to be discriminating in their choice of mating partners (Bateman, 1948). In hermaphrodites, in which individuals act simultaneously as males and as females during mating, the divergent interests over paternity seem to be even pronounced (Charnov, 1979) and are assumed to account for the evolution of special adaptations. Rather than avoiding being inseminated, individuals digest sperm and thereby reduce the competitive ability of an ejaculate (Greeff & Michiels, 1999). This should select for increased sperm numbers as well as structures to protect sperm, i.e., spermatophores (Vreys et al., 1997). In snail species without a diverticulum, the spermatophore is directly deposited in the digesting bursa copulatrix (Barker, 2001). Until the spermatophore wall is dissolved, spermatozoa have the chance to escape through the spermatophore tail and to move into the spermatheca. However, if the spermatophore is placed in a diverticulum, spermatozoa are either transported into the bursa reservoir by the strong unidirectional peristalsis of the bursa stalk or they may reach the spermatheca. Hence, we propose that the bursa tract diverticulum in *A. arbustorum* and potentially in other stylommatophoran snail species may have evolved as a countermeasure to the sperm-protecting spermatophore, in order to allow the female function to stay in control over sperm storage. Moreover, during spermatophore digestion the recurrence of mating may be inhibited (Giusti & Andreini, 1988). Therefore, the increased efficiency of digestion might also function to shorten the interval until the next spermatophore can be received.

The length of the diverticulum is also of importance because the distance spermatozoa have to move to the sperm stores increases with increasing diverticulum length. The positive allometry of diverticulum length with snail size in our study population is characteristic for traits under directional sexual selection (cf. Lüpold et al., 2004). Moreover, consistent with previous studies in *A. arbustorum*, and other helicid snails (Madec & Guiller, 1994; Baminger & Haase, 2000; van Osselaer & Tursch, 2000), we found a high phenotypic CV for diverticulum length, which is in accordance with the suggestion that sexually selected traits frequently exhibit a high CV (Pomiankowski & Møller, 1995). Because spermatozoa are most successful at reaching the storage site when the spermatophore's tail is directly protruding into the vaginal duct (Lind, 1973), the positive allometry and the increased trait variation could be the result of directional selection caused by antagonistic coevolution between diverticulum length and spermatophore length. Indeed, a comparative study across land snail species provides indications of counter-adaptation between diverticulum presence, relative length and placement and flagellum length (spermatophore's tail; Koene & Schulenburg, 2005).

In conclusion, our results suggest that the bursa tract diverticulum in *A. arbustorum* has evolved in response to selection pressures imposed by divergent evolutionary interests between male and female function. Nevertheless, more studies are pivotal to conclusively demonstrate the proposed adaptive function.

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GENERAL DISCUSSION AND OUTLOOK

Postcopulatory sexual selection is important for the evolution of the large diversity of reproductive traits found across animal taxa, even in simultaneously hermaphroditic species where animals have the inevitable task of being a father and a mother at the same time (Schilthuizen, 2005). The goal of this thesis was to investigate which factors may have influenced the occurrence and complexity of reproductive characters in the group of stylommatophoran gastropods, and whether correlated evolution due to sexual selection and sexual conflict occurs between male and female traits. Furthermore, new insights into the adaptive functions of organs related to sperm storage and sperm digestion could be gained.

The results indicate that female sperm storage organs have originated in the carrefour of snails and slugs more than once, and also have been secondarily lost several times (Chapter I). Furthermore, an extensive evolutionary diversification occurred in several groups of stylommatophoran gastropods. These results are in accordance with studies in species with separate sexes (e.g. Eberhard, 1985; Pitnick et al., 1999) and indicate that equally strong selective forces are driving reproductive trait divergence in simultaneous hermaphrodites. Moreover, comparable to the suggestion of sexual conflict promoting correlated evolution between love darts and spermatophore-receiving organs (Koene & Schulenburg, 2005), sperm storage organs may also be involved in the cycle of sexually antagonistic coevolution. The presence of complex sperm storage organs enhances female-mediated control over fertilization processes and is thus in conflict with male adaptations such as the love dart that aim to manipulate the amount of sperm stored. Thus, the positive association between complex spermathecae and the presence of dart-shooting and other kinds of auxiliary copulatory organs can be regarded as morphological counteradaptations, driven by differences in the interest of male and female function.

The mating system of snails and slugs varies strongly from almost exclusive self-fertilization over mixed mating to predominant cross-fertilization and should strongly influence reproductive morphologies. It is suggested that the absence of postcopulatory sexual selection in predominantly selfing species leads to a reduction or loss of specialized sperm storage organs, because selection should limit the size of organs such that they show a functional fit. Indeed, no spermatheca was present in selfing species and also in many species with a mixed mating system (Chapter I). In contrast, complex spermathecae were

coupled with cross-fertilization as the predominant mating system (Chapter I). Multiple mating and selective sperm use may benefit individuals by increasing the fitness of the offspring, e.g. 'by trading' up to better-quality mates, increasing the genetic quality of offspring or avoiding the use of sperm from genetically incompatible partners (Kempenaers et al., 1992; Tregenza & Wedell, 1998; Baer & Schmid-Hempel, 1999). Therefore, spermathecae with a large number of tubules, which may allow for the spatial separation of sperm from different mating partners and thus to exert cryptic female choice, might be favoured by selection (Hellriegel & Ward, 1998). This is further supported by the finding of intraspecific variation in the number of spermathecal tubules in *Arianta arbustorum* in Chapter II of this thesis and in other studies (Haase & Baur, 1995; Baminger & Haase, 1999; Baminger et al., 2000; Bojat & Haase, 2002). However, phylogenetic constraints may also have led to the pattern of spermatheca presence and complexity found across stylommatophoran species. Therefore, more detailed studies are needed also including other estimates of selection pressures, e.g. mating rate or population density.

Not only complexity, but also the size of female sperm storage organs seems to be under intense selection pressures. A correlated evolution between sperm storage organ length and sperm length, presumably driven by postcopulatory sexual selection, has been revealed in many taxa of gonochorists (e.g. Dybas & Dybas, 1981; Briskie & Montgomerie, 1992; Pitnick et al., 1999; Presgraves et al., 1999; Minder et al., 2005). Similarly, the size of storage organs across the hermaphroditic stylommatophoran gastropods was correlated with the size of sperm (Chapter I). This relationship seems to result from the way in which sperm and spermathecae interact. In *Drosophila*, longer sperm have a fertilization advantage by occupying and/or retaining occupancy in the storage organ better, or by being better in displacing or resisting being displaced by shorter sperm (Pattarini et al., 2006). For stylommatophorans it has been suggested that the unified beating of the flagella of sperm from the first mate could provide paternity assurance through increased resistance to incoming sperm from subsequent mates entering the tubules (Rogers & Chase, 2002). Therefore the large sperm size found in many species may be advantageous, but furthermore the number of sperm reaching the storage organ could be important for filling the storage organ and blocking it for future mates. This fact may explain the positive relationship found between the number of sperm transferred and sperm length across different populations of *A. arbustorum* (Chapter II), which is contrary to Parker's (1982) prediction of a trade-off in sperm investment. Moreover, because larger

sperm storage organs could allow the female function to take up more sperm and/or to select sperm depending on their quality and thereby to benefit from multiple mating and a greater control over the fertilization process (Eberhard, 1996; Pitnick et al., 1999; Miller & Pitnick, 2002), a conflict with the male interest of siring as many offspring as possible may arise. This conflict over the processes involved in sperm storage and sperm use may set off an coevolutionary arms race between male and female function (Rice, 1996). Potentially driven by this arms race, the volume of the spermatheca and the number of sperm transferred positively correlate across six populations of *A. arbustorum*, although in this species no correlated evolution between spermatheca length and sperm length could be revealed (Chapter II).

Previous studies indicate that also the physical properties of the sperm storage organ could influence sperm utilization patterns (Walker, 1980; Simmons, 1986). The spermatheca of *A. arbustorum* increases following a single mating through an expansion of the tubule diameter and may accommodate more sperm than would be expected from measuring its initial volume (Chapter III). This bears important consequences for cryptic female choice because the storage of successive ejaculates may enable the female function to use sperm of more than one mating partner for fertilization (Simmons, 2001). Furthermore, the number of sperm transferred was not related to the amount of sperm reaching the storage organ and the amount of allosperm digested was not related to the size of the received spermatophore in *A. arbustorum* (Chapter III). Both findings also suggest a female influence on the processes of sperm storage, potentially through selective digestion of different amounts of sperm received from the mating partner. These observations could, nevertheless, also be the result of different sperm quality (swimming speed, longevity) of the sperm donors (e.g. Garcia-Gonzalez & Simmons, 2005; Minoretti & Baur, 2006).

There are indications that several other reproductive characters of stylommatophoran gastropods are involved in the coevolution, as noted for love darts earlier in this discussion (Schilthuizen, 2005). Further traits include the flagellum, which produces the spermatophore's tail, and the diverticulum, which receives the spermatophore during mating. A comparative study across snail species already suggested counter-adaptations between diverticulum presence, relative length and placement and flagellum length (Koene & Schulenburg, 2005). Consistent with this, positive associations between a complex spermatheca and a long flagellum and between spermatheca absence and flagellum as well as diverticulum absence were found (Chapter I).

These results demonstrate how different sets of male and female reproductive traits are linked with each other and that it is important to consider as many characters as possible in comparative analyses. However, it further complicates disentangling which traits directly influence the selection on other traits, especially if reproductive tracts are as differentiated as in stylommatophoran gastropods. A better understanding of the adaptive function of reproductive organs is therefore needed. Nevertheless, beside detailed studies dealing with the function of dart-shooting in helicid snails on the one hand (Koene & Chase, 1998; Landolfa, 2002; Chase & Blanchard, 2006), and the sperm digesting bursa copulatrix (Németh & Kovács, 1972; Rogers et al., 1980; Gomez et al., 1991) on the other hand, no deeper investigations of the diverse morphological adaptations in the reproductive tract of stylommatophoran gastropods exist. Similarly, the bursa tract diverticulum, although used as a trait in comparative studies (Koene & Schulenburg, 2005), has received no particular attention. In this thesis, however, it could be shown that the diverticulum of *A. arbustorum* serves, beside the bursa copulatrix, as a site of spermatophore digestion or at least for the partly break down of spermatophores (Chapter IV). Furthermore, the positive allometry and the high phenotypic variation of diverticulum length in comparison to snail size are characteristic for traits under directional sexual selection (cf. Lüpold et al., 2004). These results suggest, together with findings from other studies, that the diverticulum evolved as a countermeasure of the female function to retain control over the fate of received sperm. Sperm digestion may have favoured the evolution of structures that protect sperm, i.e., spermatophores (Vreys et al., 1997). In species without a diverticulum, the spermatophore is directly deposited in the bursa copulatrix (Barker, 2001). Until the spermatophore wall is digested sperm have the chance to escape and move to the sperm storage organ. With a diverticulum, however, sperm leaving the spermatophore are either transported into the bursa reservoir for digestion or may reach the spermatheca. This mechanism allows the selective digestion or storage of sperm and may answer the question of why a diverticulum occurs as a second digestive organ in some helicid snail species.

The majority of studies in this thesis revealed patterns of reproductive trait evolution due to postcopulatory sexual selection. However, we also found associations of carrefour complexity with several life-history traits, including body size, reproductive strategy (semelparity vs. iteroparity) and reproductive mode (oviparity vs. ovoviviparity), as well as with particular habitats (Chapter I). These results suggest that additional factor may select for the presence of specialized sperm storage organs and possibly other morphological characteristics. Therefore, it remains a challenge to separate the effects of

the diverse selection pressures on reproductive tract morphologies in stylommatophoran gastropods.

The realization that sexual selection and sexual conflict play an important role in hermaphrodite evolution has promoted a young field of studies on reproductive trait divergence in the species-rich group of stylommatophoran snails and slugs with their bizarre and enigmatic sexual structures and behaviours. This thesis adds new information on factors that could have shaped male and female reproductive morphology in *A. arbustorum* and other stylommatophoran gastropods. Nevertheless, still much knowledge is needed to understand the pattern of trait divergence in the context of postcopulatory sexual selection and sexually antagonistic coevolution and many open questions remain to be answered.

"Having two sexes makes hermaphrodites twice as interesting" (Schilthuizen, 2005).

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AUTHOR'S ADRESSES

Georg Armbruster:

Department of Environmental Sciences
Section of Plant Ecology
Schönbeinstrasse 6
CH-4056 Basel
Switzerland

Bruno Baur, Kathleen Beese:

Department of Environmental Sciences
Section of Conservation Biology (NLU)
University of Basel
St. Johanns-Vorstadt 10
CH-4056 Basel
Switzerland

Konstantin Beier:

Anatomical Institute
University of Basel
Pestalozzistrasse 20
CH-4056 Basel
Switzerland

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CURRICULUM VITAE

NAME	Kathleen Beese
DAY OF BIRTH	26/01/1977
PLACE OF BIRTH	Friedrichroda
NATIONALITY	Deutschland
ADDRESS	Kreuzstrasse 18, 4600 Olten
CIVIL STATUS	married to Bastian Jentsch

EDUCATION	1983 – 1991	Comprehensive school "POS Hörselgau"
	1991 – 1995	Gymnasium "Salzmannschule Schnepfenthal"
UNIVERSITY EDUCATION		
Basic:	1995 – 1997	Biology, Friedrich Schiller University Jena, Germany
Higher:	1997 – 1999	Systematic Zoology, Neurobiology, Anthropology, Ecology, FSU Jena, Germany
Diploma thesis:	1999 – 2001	"Strukturelle Flexibilität des Verdauungstraktes von Rotseitenstrumpfbandnattern (*Thamnophis sirtalis parietalis*)", Department of Systematic Zoology and Evolutionary Biology, FSU Jena, Germany; Supervisor: PD Dr. J. M. Starck, Co-referee: Prof. M. S. Fischer
	2002	Guest Researcher at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, Field research in Ivory Coast, Westafrica
Doctoral Thesis:	2003 – 2007	"The evolution of male and female reproductive traits in simultaneously hermaphroditic terrestrial gastropods", Department of Environmental Sciences, University of Basel, Switzerland, Supervisor: Prof. B. Baur, Co-referee: PD Dr. A. Erhardt
WORKING EXPERIENCE	1998 – 2006	Teaching assistant for basic zoological courses and zoological and ecological field courses, FSU Jena, Germany and University of Basel, Switzerland
	2000 – 2001	Congress secretary for the 6th International Congress of Vertebrate Morphology (ICVM-6), Jena, Germany, 07/2001
	2000 – 2001	Tutorial for systematic zoology lecture, FSU Jena, Germany

ORAL PRESENTATIONS

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GRANTS RECEIVED

2005 Fonds zur Förderung des wissenschaftlichen Nachwuchses (travel grant) to attend the Symposium: Sexual selection and "primary" sexual characters at the 29th International Ethological conference, Budapest, Hungary: CHF 870

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