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Intra-specific morphological variation of the spermatheca in the simultaneously hermaphroditic land snail *Helix aperta*

Meriem Abdelli[1,2] · Saida Benbellil-Tafoughalt[1] · Joris M. Koene[2]

Abstract In the majority of internally fertilizing animals, females are equipped with sperm storage organs where they store the sperm received during copulation. In many simultaneously hermaphroditic pulmonates, these organs consist of complex spermathecae that show inter- and intra-specific variation in their structure. This variability is theoretically predicted by postcopulatory sexual selection in the context of sperm competition and cryptic female choice. In this study, the variation in the structure of the spermatheca was investigated in the land snail *Helix aperta* from four natural populations near Bejaia in northern Algeria. The populations were different in local snail density, probably also reflecting the intensity of sperm competition. We tested whether the spermatheca showed differences that are predicted by sperm competition theory. In addition, we tested whether the spermathecal structure depends on the shell size and/or is correlated with other reproductive organs that are thought to be affected by sexual selection. We found that the fertilization pouch of *H. aperta* consists of a simple fertilization chamber and 3–9 spermathecal tubules. The four populations did not differ significantly in the mean number of these tubules.

However, significant differences were found in the length of the main tubule, the length of the fertilization chamber, and the average length of lateral tubules. In addition, strong associations were detected between the lengths of these structures and the local snail density, while no effect of shell size or reproductive organs was found. Our results indicate that the intensity of sperm competition may not affect the total number of spermathecal tubules, but may increase their lengths. This increase in spermathecal length may reflect an improved sperm storage capacity that is probably beneficial in situations of high sperm competitions intensity.

Introduction

Sperm storage is a common phenomenon in many animals including earthworms, molluscs, arthropods, birds, reptiles, amphibians, fish, and mammals (Birkhead and Møller 1993; Holt 2011; Orr and Zuk 2012). It is generally defined as the retention and preservation of the sperm received during copulation inside the female reproductive tract for an extended period of time (Orr and Zuk 2012, 2014) that can range from weeks to months and even years before being used for fertilization (e.g., Parker 1970; Pamilo 1991; Sever et al. 2001, 2003; for reviews, see also Neubaum and Wolfner 1999; Suarez 2008). This process seems to be the result of the timing of insemination and ovulation no longer being synchronized in females of some animals (reviewed in Sasanami et al. 2013), the diversity of their life histories, scarcity of mating opportunities, or simply the need to avoid repeated and possibly unsafe matings that can

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experience many animals (reviewed in Holt 2011). As a result, in many species, sperm storage constitutes an integral part of their reproductive strategy.

Sperm storage clearly provides several advantages. For example, the prolonged storage of received sperm allows for assurance of reproductive success and a lengthening of the breeding season (see Conner and Crews 1980; Sever and Brizzi 1998). In addition, it can help in minimizing the number of matings, and thus any costs associated with that, and can also facilitate colonization (reviewed in: Neubaum and Wolfner 1999; Eckstut et al. 2009; see also: Kalb et al. 1993; Tram and Wolfner 1999; Liu and Kubli 2003; Peng et al. 2005). Furthermore, sperm storage implies protection for the sperm, thus sustaining their viability for long periods (e.g., Ribou and Reinhardt 2012). Besides the summary above, more details about female sperm storage advantages have been reviewed previously by many authors (e.g., see Neubaum and Wolfner 1999; Bloch Qazi et al. 2003).

To store sperm, many species have developed dedicated structures in their reproductive tract. These structures range from more or less complex tubules (e.g., in birds: Bakst 1987; Birkhead and Møller 1992; Brillard 1993; Sasanami et al. 2013; Holt and Fazeli 2016 and some reptiles: Pearse and Avise 2001; Han et al. 2008) to highly sophisticated organs containing multiple compartments (Eberhard 1985, 1996; e.g., the spermathecae of amphibians: Sever 1991, 1997, 2002; insects: Bloch Qazi et al. 1998; Baer et al. 2006; Córdoba-Aguilar et al. 2003 and gastropods: Baminger and Haase 1999; Koemtzopoulos and Staikou 2007; Whelan and Strong 2014; annelids: Novo et al. 2013). Interestingly, large interspecific divergence in morphology, size, complexity, and even number of these organs have been reported. Hypothetically, these differences may be explained by the large variation in sperm size and shape (e.g. Walker 1980; Keller and Reeve 1994; Pitnick et al. 1995; Minoretti and Baur 2006) and the differences in life history traits and/or habitat specificity occurring among such species (reviewed in Pitnick et al. 1999; Beese et al. 2008).

Another widely supported explanation supposes that the sperm-storage organs' morphology and/or number evolve in response to postcopulatory sexual selection (e.g. Hellriegel and Ward 1998; Pitnick et al. 1999). In such cases, multiple matings take place and multiple ejaculates overlap within the female tract prior to egg production, thus enhancing sperm competition (i.e., establishment of the conditions outlined by Parker 1970, 1984). Clearly, the latter introduces variation in male reproductive success and leads to postcopulatory intra-sexual selection, thus promoting morphological, physiological, and behavioural traits that may increase the fertilization success of an ejaculate under competitive conditions (Pizzari and Parker 2009). In contrast, given that females are generally the sex

that invests more in reproduction (Parker 1984), they are also expected to try to control the outcome of sperm competition in favor of the sperm of certain males they copulated with. They can potentially do this by selectively using sperm, a mechanism known as cryptic female choice (Eberhard 1996). In the light of this hypothesis, the evolution of sperm-storage organs can also be explained as a female adaptation that allows for greater control over offspring paternity (Eberhard 1996).

In the context of sperm storage, the pulmonate land snails (Stylommatophora) are an interesting group to study, as they are known to mate promiscuously and are hermaphroditic. Moreover, they are known to store sperm for long periods of time (Baur 1998) and some possess rather sophisticated spermatheca (see Tompa 1984, Beese et al. 2008). In these species, the spermatheca forms part of a more or less complex system of tubules of an organ often referred to as fertilization pouch or fertilization pouch–spermatheca complex (Tompa 1984). The structure of the fertilization pouch has been studied in many gastropods (Tompa 1984; Beese et al. 2008) with less attention paid to land snails (reviewed in Koemtzopoulos and Staikou 2007), except of two species: *A. arbustorum* (Haase and Baur 1995; Baminger and Haase 1999; Beese et al. 2006) and *C. aspersum* (Koemtzopoulos and Staikou 2007).

In general, a land snail's fertilization pouch consists of a fertilization chamber and a spermatheca, which can be made up of a variable number of tubules (e.g., one tubule in *Bradybaena fructicum*, Schileyko and Schileyko 1992; Bojat et al. 2002; two in *Succinea putris*, Rigby 1965: 34 in *Drymaeus papyraceus*, van Mol 1971). The number of spermathecal tubules also varies within species (e.g., 3–5 tubules in *H. pomatia*, Lind 1973; 2–9 in *A. arbustorum*, Baminger and Haase 1999; 1–8 in *Cepaea vindobonensis*, Staikou 2001; and 5–16 in *Helix lucorum* Staikou 2001). The tubular structure of the spermatheca has been suggested to allow for spatial segregation of the sperm from different partners, thus offering the opportunity for cryptic female choice (Haase and Baur 1995). The only suggestive evidence for such an ability, however, comes from ultrastructural investigations of the epithelium and the surrounding musculature of the spermatheca of *A. arbustorum* (Bojat et al. 2001a, b). Since postcopulatory sexual selection, in the form of sperm competition and cryptic female choice, seems to be a strong evolutionary force, it is likely to be accompanied by a male–female coevolution of other behavioural and anatomical traits related to reproduction (Arnqvist and Rowe 2002; Pitnick et al. 2003). Indeed, the presence and complexity of sperm-storage organs has been shown to co-evolve with other reproductive organs in snails (see Beese et al. 2008).

The variation in spermathecae cited above has been predicted to be mainly influenced by the level of sperm competition and is thus expected to result in increased

complexity of the spermatheca, for example in terms of number of tubules, under more intense sperm competition (reviewed in Baminger and Haase 1999; Koemtzopoulos and Staikou 2007). However, the empirical studies undertaken by the same authors investigating the relation between the complexity of the spermatheca and density, as a proxy for sperm competition intensity, among different populations of *A. arbustorum* and *C. aspersum* did not reveal any correlation. Given that this was only tested in two species so far, in the present study, we decided to focus on the simultaneously hermaphroditic land snail *H. aperta*.

We started out by examining and describing, for the first time, the fertilization pouch and spermatheca of this species. Subsequently, we investigated the morphological variation in the spermatheca of this species in four different populations that differed in snail densities, using the latter as a proxy for sperm competition intensity. While this is not a direct measure of sperm competition, in many hermaphroditic animals, increases in density result in more frequent multiple matings (Michiels 1998), so we assume here that a higher population density also represents more frequent mating. Finally, we tested for correlations between the morphology of the spermatheca with other male (epiphallus, flagellum, dart sac, and digitiform glands) and female (diverticulum and bursa stalk) reproductive organs. We chose to focus on these particular organs, because they are thought to be implicated in postcopulatory sexual selection and could thus be expected to interact with the morphology of the sperm-storage organ (Baminger and Haase 2000; Davison et al. 2005; Koene and Schulenburg 2005).

Materials and methods

Study organism

Helix aperta, sometimes also referred to as *Cantareus apertus* (Born 1778) or *Helix naticoides* (Draparnaud 1801), is a circum-mediterranean hermaphroditic land snail species, generally distributed in the south of France, Turkey, Cyprus, and North Africa (Kerney and Cameron 1979; Schütt 2001). In Algeria, it is frequently encountered in the coastal part of the country, especially in the region of Kabylia (Benbellil-Tafoughalt et al. 2009). This species is also introduced in America (California and Louisiana), New Zealand, and Australia (Kerney and Cameron 1979; Schütt 2001). The shell of this species is spherical, of a moderate adult size (on average 27 ± 4 mm) and without any banding (Yildirim 2004). The body color of these snails varies from greenish yellow to dark green or black according to the lifestage of the animal (Benbellil-Tafoughalt et al. 2009). In its Mediterranean and coastal

habitats, it is adapted to live on the soil, among grasses and with a preference for vineyards and olive orchards (Giusti and Andreini 1988). In general, it does not occur above altitudes of 900 ma.s.l. (Germain 1930, 1931). The activity of this species is very much dependent on environmental conditions such as temperature, photoperiod, and humidity (Benbellil-Tafoughalt et al. 2009, 2011; Benbellil-Tafoughalt and Koene 2015). Hence, it can spend long periods of aestivation or hibernation underground when conditions are not right. Under such circumstances, it burrows 7–15 cm deep, closes off its shell with a thick whitish epiphragm, and enters into a state of metabolic slowdown. As a result of this habit, it is known as the "burrowing snail". Morphologically and phylogenetically, this species is closely related to *Cornu aspersum* (Germain 1930, 1931; Ktari and Rezig 1976; Koene and Schulenburg 2005). *H. aperta* from Italy and northern Algeria were reported to preferably mate in autumn, which in its investigated habitats means from the beginning of October up to the second half of December, with temperatures around 20 °C and long days (16 h L: 8 h D) (Giusti and Andreini 1988; Benbellil-Tafoughalt et al. 2011). However, in Tunisia, they were found to mate at the end of summer under short-day conditions (6 h L: 18 h D; de Vaufleury and Gimbert 2009). Benbellil-Tafoughalt et al. 2009 (see also Benbellil-Tafoughalt and Koene 2015) also revealed that reproduction and growth are strongly affected by the length of the photoperiod as well as temperature.

Population sampling

Samples of 10–18 adult *Helix aperta* were randomly collected during rainy weather conditions at four different localities (referred to as populations) from the region of Bejaia in northern Algeria. The sampling was done at the beginning of February 2016 within 2 days to avoid any influence of seasonal variation. Two populations came from irrigated agricultural monocultures, situated in a rural coastal region at Baccaro (36°39′6.10″N, 5°12′5.09″E) and an exposed rural region at Tala Hamza (36°41′43.49″N, 4°59′17.49″E). The third population came from an abandoned garden in an urban area at "place du Stade" (36°45′1.36″N, 5°2′42.97″E) that was covered with ferns, bushes, and clovers, while the fourth population came from an exposed natural meadow in front of an urban area at Sidi Ahmed (36°45′26.10″N, 5°3′53.40″E) and was densely covered with grasses, clovers, and sparse shrubs.

The density of each population was expressed as the number of adult snails/m^2 and estimated by means of random quadrat sampling (0.25×0.25 m). The estimation was performed once at the end of autumn during the selection of suitable populations for the study (early December 2015). At that time, *H. aperta* emerges from

aestivation and is active above ground. To obtain a reliable measure, we adopted the Elliot's method (1971) to determine the necessary number of sampling units per site for an error lower than 20%.

Measurements and histology

The collected snails were transported to the laboratory on the same day and their shell's maximum diameter and height were measured using Vernier calipers with an accuracy of 0.1 mm. After subsequent anesthesia by injection of 50 mM $MgCl_2$ via the dorsal side of back of the foot and fixation in 70% ethanol, we removed the shell of each snail and dissected out the distal parts of its genitalia (excluding two dissections where some organs were ruptured). For each individual, the following anatomical reproductive traits were measured to the nearest 0.1 mm using Vernier calipers: "male tract organs" (dart sac, digitiform glands, epiphallus, and flagellum) and "female tract organs" (bursa copulatrix tract/bursa stalk and diverticulum). Before measuring, each structure was extended straight without any convolutions in a petri dish with distilled water and its length was then measured three times. The mean (for digitiform glands, the mean of the mean of both glands) was then calculated and used for statistical analyses. Although the presence of these organs has been established for *H. aperta* (Giusti and Andreini 1988), no quantitative measurements of their size are available so far, while their function is assumed to be similar to those in related species.

The fertilization pouches, sometimes together with small parts of spermoviduct and hermaphroditic duct, were dissected out and fixed in Davidson's Fixative (AFA fixative) for 24 h. Because the fertilization pouch of *H. aperta* turned out to be relatively deeply embedded in the albumen gland, some remains of this gland were also always dissected along. Fertilization pouches where embedded in paraffin, serially sectioned at 7 μm and stained with Hematoxylin–eosin. The sections were always made along the cross-sectional plan of the organ. The structure of each spermatheca was examined by counting the number of spermathecal tubules and observing their branching pattern. The length of each tubule was approximated by counting the number of cross sections in which it appeared, starting from the section in which the tubule was clearly separated from the tubule from which it branched off, and multiplying it by the section thickness (7 μm). The length of the fertilization chamber was estimated for each individual in the same way.

Statistical analyses

We used one-way ANOVAs or Kruskal–Wallis tests to test for possible differences in shell size (height and diameter)

and spermathecal structures (length and number) among the populations.

To study the effects of population density and shell size (diameter) and their interaction on spermathecal structures and the reproductive organs considered, we used a linear regression model for response variables that were normally distributed. In this model, density and diameter were considered as fixed factors, while the spermathecal structures and reproductive organs were used as response variables. For non-normally distributed response variables, we used a generalized linear model with the same fixed factors. Note that we chose to use diameter as the representative measure of body size, which is widely used in pulmonates (reviewed in Baminger and Haase 2000), but the same models were also run with shell height as a fixed factor instead.

Finally, possible relationships between spermathecal structures and the reproductive organs, as well as between the spermathecal structures themselves, were investigated through Pearson or Spearman correlations.

Statistical analyses were performed using Xlstat 2009 and JMP 9. All variables were initially tested for normality and non-parametric tests were used when needed. Means and standard deviations (± SD) are reported unless otherwise stated.

Results

Our histological observations revealed that the fertilization pouch of *H. aperta* is a complex organ located in the distal part of the reproductive tract. We found that it consists of a blind-ended fertilization chamber into which the hermaphroditic duct and the spermatheca open (see Fig. 1). The fertilization chamber is the longest structure of the fertilization pouch and appears more or less c-shaped in the cross sections. According to Haase and Baur (1995), this is the site where the eggs are fertilized by the sperm stored in the spermatheca. We found that the spermatheca consists of several tubules with one common entrance (Fig. 1). The longest tubule, which always branched off the fertilization chamber, was considered as the "main tubule"
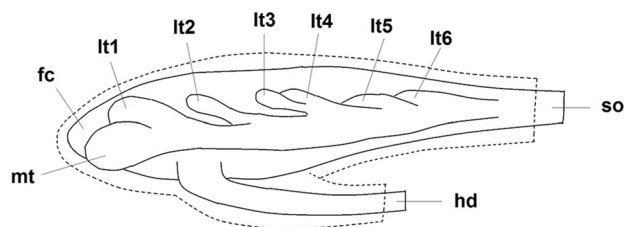

Fig. 1 Schematic drawing of the fertilization pouch of *H. aperta*: *fc* fertilization chamber, *mt* main spermathecal tubule, *lt* lateral spermathecal tubules (numbered 1–6); *hd* hermaphroditic duct, *so* spermoviduct

(conforming to earlier descriptions in *A. arbustorum* Baminger and Haase 1999 and *C. aspersum* Koemtzopoulos and Staikou 2007). All other tubules were considered as "lateral tubules" (see Baminger and Haase 1999; Koemtzopoulos and Staikou 2007). The latter either branched off from the main tubule or from other lateral tubules. In 14% of the spermathecae, some lateral tubules were seen to branch off from the fertilization chamber directly at the same point where the main tubule branched off. This pattern was observed in all four populations. The lengths of tubules were found to vary considerably within individuals. The main tubule was always shorter than the fertilization chamber. The number of spermathecal tubules also varied, ranging from 3 to 9 across the different populations studied. The four populations did not differ significantly in the number of spermathecal tubules (Kruskal–Wallis, $H = 7.815$, $P = 0.668$ see Table 1).

Interestingly, the length of the fertilization chamber (Kruskal–Wallis, $H = 8.25$, $N = 52$, $P = 0.041$), the length of the main tubule (ANOVA, $F = 4.73$, $P = 0.006$, $df = 3$, 48), and the average length of lateral tubules (Kruskal–Wallis, $H = 38.63$, $N = 52$, $P < 0.0001$) were seen to vary significantly among the four populations (see Table 1 and Fig. 2 for post hoc test results). Moreover,

ANOVAs revealed significant differences in shell height ($F = 9.581$, $P = 0.0001$, $df = 3$, 48) and diameter ($F = 5.715$, $P = 0.002$, $df = 3$, 48) between the populations (see Table 1). The mean shell height was significantly correlated with the mean shell diameter (Pearson correlation, $r = 0.653$, $N = 52$, $P < 0.0001$) over the four populations. In addition, no significant differences were detected in the length of any of the reproductive organs considered (see ANOVA results in Table 1).

The populations' local snail density ranged from 3.6 to 22.4 adult individuals/m^2 and was inversely correlated with shell diameter (Spearman rank correlation, $r = -0.467$, $N = 52$, $P = 0.001$). However, it was not correlated with any of the reproductive organs studied (Spearman rank correlation, all $P > 0.05$, see Supplementary Table 1).

The linear regression models investigating the dependence of the spermathecal structures on shell size and population density are summarized in Table 2. Only the populations' local density had a significant effect on the length of the fertilization chamber, the length of the main tubule, and the average length of lateral tubules (see Table 2). All three increased with increasing population density. In addition, neither the density nor the shell size (diameter) affected the number of spermathecal tubules.

Table 1 Comparison of shell size, reproductive organs, fertilization chamber length, and both number and length of spermathecal tubules of four populations of *H. aperta* using either ANOVA or Kruskal–Wallis tests (respectively, *H*- and *F*-values indicated)

	Population				F or H	P
	Stade	Sidi Ahmed	Tala Hamza	Baccaro		
N	8	15	18	11		
Density	3.6	7.2	18	22.4		
Shell size (mm)						
Height	25.02 ± 0.5	26.93 ± 0.63	23.90 ± 0.53	22.65 ± 0.57	9.58	< 0.0001
Diameter	21.12 ± 0.42	21.87 ± 0.62	20.25 ± 0.44	18.75 ± 0.54	5.71	0.002
Male tract organs (mm)						
Dart sac	9.27 ± 0.65	9.78 ± 0.41	9.62 ± 0.29	8.93 ± 0.24	0.93	0.432
Epiphallus	11.73 ± 1.44	12.32 ± 0.80	13.80 ± 0.59	11.51 ± 0.98	1.60	0.202
Flagellum	17.95 ± 1.16	21.35 ± 0.91	19.82 ± 0.76	18.24 ± 1.29	2.33	0.086
Digitiform glands	5.68 ± 0.97	6.27 ± 0.53	6.57 ± 0.32	6.49 ± 0.53	0.44	0.728
Female tract organs (mm)						
Diverticulum	38.14 ± 4.63	44.00 ± 3.11	37.11 ± 2.67	34.36 ± 2.91	1.70	0.179
Bursa stalk	16.46 ± 1.88	16.85 ± 0.95	15.18 ± 0.72	15.50 ± 0.89	*0.68*	0.567
Fertilization chamber length (mm)	1.86 ± 0.11	1.92 ± 0.05	2.19 ± 0.10	2.22 ± 0.11	*8.25*	0.041
Spermathecal structures						
Number of tubules	5.5 (4–9)	6 (3–8)	5 (3–8)	6 (3–9)	*1.56*	0.668
Length of main tubule	1.48 ± 0.09	1.53 ± 0.09	1.95 ± 0.09	1.80 ± 0.14	4.73	0.006
Average length of lateral tubules	0.46 ± 0.14	0.57 ± 0.05	1.02 ± 0.19	0.73 ± 0.16	*8.63*	< 0.0001

Descriptive statistics (mean ± SE) are presented for each population. Information about the sampling sites is mentioned in the "Materials and methods"
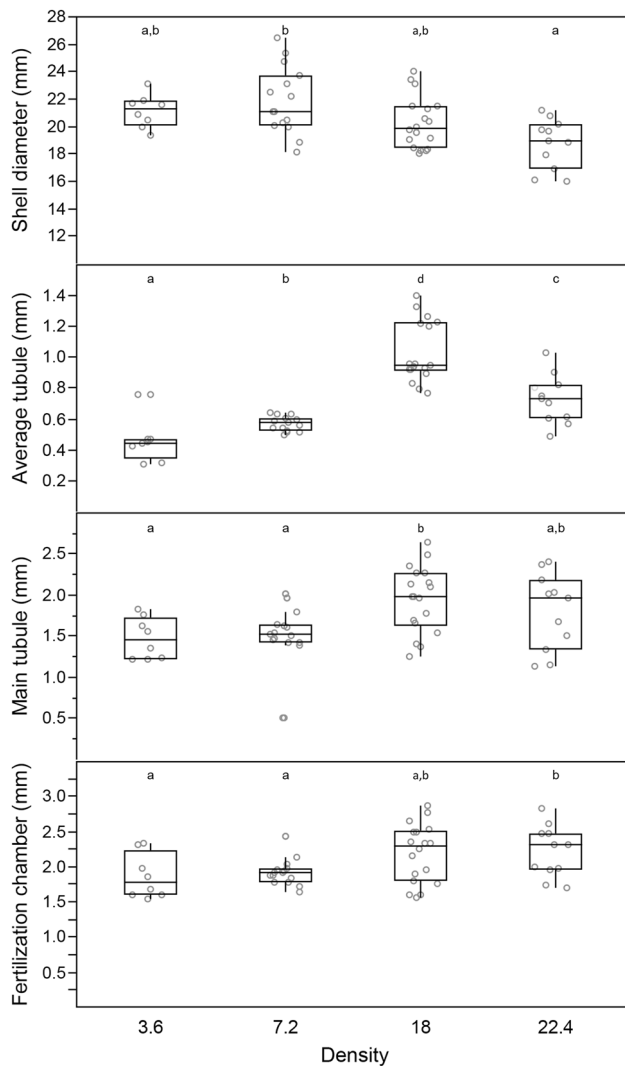
H values are indicated in italic

Fig. 2 Comparison of shell diameter and the lengths of the fertilization chamber, main tubule, and average lateral tubules between the four populations of *H. aperta* with different local densities. Boxplots show the median, the 1st and 3rd quartiles, and the range. The small circles indicate the individual data points. The letters indicate significant differences between the densities based on parametric Tukey or non-parametric Wilcoxon multiple comparison post hoc testing ($P < 0.05$; see "Results")

Similarly, no significant correlations were found between any of the reproductive organs considered in this study and the length or number of the spermathecal structures (all $P > 0.06$, see Supplementary Table 1).

Significant correlations were found between the lengths of most spermathecal structures (all $P < 0.040$, see Supplementary Table 1). The only non-significant correlations were the ones between the number of spermathecal tubules and both the length of the fertilization chamber (Spearman: $r = 0.070$, $N = 52$, $P = 0.621$) and the length of the main tubule (Spearman rank correlation: $r = 0.033$, $N = 52$, $P = 0.817$), while a negative correlation was found

between the average length of the lateral tubules and the number of spermathecal tubules (Spearman rank correlation: $r = -0.407$, $N = 52$, $P = 0.03$).

Discussion

This study provides the first description of the fertilization pouch–spermatheca complex of the simultaneously hermaphroditic land snail *H. aperta*. The overall morphological structure of these organs turns out to be fairly similar to those of other Stylommatophora land snails (e.g., *A. arbustorum*: Haase and Baur 1995; Baminger and Haase 1999; *C. aspersum*: Evanno and Madec 2007; Koemtzopoulos and Staikou 2007). We also found same morphological pattern among the four populations, which agrees with the previous findings on *C. aspersum* that reported a consistent spermatheca structure even over consecutive generations, indicating that this has a genetic basis (Koemtzopoulos and Staikou 2007). Similar to the studies of Baminger and Haase (1999) and Koemtzopoulos and Staikou (2007), we could distinguish a main tubule and a number of lateral tubules. The main tubule, defined as the longest tubule in the spermatheca, was always shorter than the fertilization chamber. This was also reported for *C. aspersum* (Koemtzopoulos and Staikou 2007), but not for *A. arbustorum* where the main tubule was longer than the fertilization chamber in most cases (Baminger and Haase 1999). In the majority of the analyzed spermathecae, the lateral tubules either branched off from the main tubule or from other lateral tubules; however, exceptions to this occurred in 14% of spermathecae where some lateral tubules branched off directly from the fertilization chamber. Although we have no biologically meaningful explanation for this, a similar pattern was reported for *C. aspersum* (Koemtzopoulos and Staikou 2007).

As expected, the total number of tubules in the spermatheca of *H. aperta* was variable (3–9 tubules) and was almost similar to that of *A. arbustorum* (2–9 tubules, Baminger and Haase 1999). Such an intra-specific variability has been previously reported in many other species (see "Introduction"). This large variation within each population may explain why we did not find the hypothesized difference in tubule numbers between the four populations, and thus densities, that we studied (see also Baminger and Haase 1999). This is consistent with earlier, similar studies on six populations of *A. arbustorum* from the Eastern Alps in Austria (Baminger and Haase 1999) and five populations of *C. aspersum* from Greece (Koemtzopoulos and Staikou 2007). While we did not evaluate the amount of sperm stored, in *A. arbustorum,* this was also found not to differ or to correlate with density (Baminger and Haase 1999). This may suggest that the

Table 2 Summary of the models used to test the effect of the fixed factors: density of each population and diameter of the shell of individuals on the measurements of the spermathecal structures and the reproductive organs (response variables)

	Density			Shell diameter			Density × shell diameter		
	df	F or χ^2	P	df	F or χ^2	P	df	F or χ^2	P
Spermathecal structures									
Number of tubules	3, 44	0.910	0.823	1, 44	≈0.001	≈1.000	3, 44	1.058	0.787
Length of fertilization chamber	3, 44	14.799	0.0020	1, 44	0.190	0.6629	3, 44	0.975	0.8072
Length of main tubule	3, 44	5.452	0.0028	1, 44	1.587	0.2144	3, 44	0.223	0.8803
Average length of lateral tubules	3, 44	101.41	< .0001	1, 44	1.0215	0.3122	3, 44	1.898	0.5938
Reproductive organs									
Diverticulum	3, 44	2.327	0.0877	1, 44	0.466	0.4983	3, 44	2.115	0.1119
Bursa stalk	3, 44	0.137	0.9871	1, 44	0.338	0.5611	3, 44	0.477	0.9240
Dart sac	3, 44	0.856	0.4713	1, 44	0.111	0.7410	3, 44	0.781	0.5108
Epiphallus	3, 44	1.030	0.3885	1, 44	0.077	0.7833	3, 44	1.306	0.2844
Flagellum	3, 44	1.023	0.3918	1, 44	0.024	0.8772	3, 44	0.995	0.4039
Digitiform glands	3, 44	0.393	0.7584	1, 44	0.0001	0.9911	3, 44	0.371	0.7741

The × in the last column indicates the tested interaction between the fixed factors

χ^2 values are indicated in italic

actual number of spermathecal tubules does not reflect the outcome of sperm competition and may not be subject to sexual selection (cf. Baminger and Haase 1999; Koemtzopoulos and Staikou 2007). Moreover, if the number of spermathecal tubules is genetically controlled or depends on an individual's age or maturity (as proposed by Koemtzopoulos and Staikou 2007 and Baminger and Haase 1999, respectively), this could also explain the absence of a correlation between population density and the number of spermathecal tubules. However, a study of the spermathecal ontogeny of individuals of *A. arbustorum* revealed that the development of lateral tubules is not simultaneous, but that these branch off successively from the main tubule, which develops first (Baminger and Haase 2002). Furthermore, and importantly, that study revealed that the final number of spermathecal tubules is already reached in sub-adult individuals (Baminger and Haase 2002). The latter indicates that the number of spermathecal tubules probably does not change after maturity is reached.

Interestingly, our data reveal a significant positive relationship between the average length of the fertilization chamber, main tubule, and lateral tubules with population density. This contradicts the findings of the previous studies on both *A. arbustorum* and *C. aspersum* (Baminger and Haase 1999; Koemtzopoulos and Staikou 2007) in which density was found not to have any effect on the length of spermathecal structures. In this context, it is noteworthy that the populations' densities in the two previous studies and the present one are clearly different (Baminger and Haase 1999: 0.9–39.8 adults/m²; Koemtzopoulos and Staikou 2007: 11.4–42.3 adults/m²), although this is probably not enough to explain the difference between the species. Of course, this could also simply be due to differences between the species, as Lodi et al. (2017/in press) also reported for other reproductive traits in land snails.

Moreover, it should be noted that in the previous studies and in the present one, snail density was used as a proxy for sperm competition intensity. Although there generally seems to be a positive relation between density or group size and sperm competition in many animals (prosobranch gastropods: Oppliger et al. 1998; insects: Gage 1995; birds: Birkhead and Møller 1992; mammals: Møller and Birkhead 1989; Hirudinea annelids: Tan et al. 2004; anuran amphibians: Buzatto et al. 2015), the only evidence that we have for land snails is the difference observed in mating frequencies among populations of *C. aspersum* from contrasting local densities, which also persisted in the F1 generation snails (Koemtzopoulos and Staikou 2007). However, density and the minimum number of estimated fathers do seem to be positively correlated in egg clutches of other snails (e.g., *Lymnaea stagnalis*: Nakadera et al. 2017). Therefore, density may reflect different sperm competition intensities in the species studied here, but this really needs to be confirmed by a paternity study. So far, we have no detailed information about multiple matings in *H. aperta* and whether mating frequency tends to increase with increasing population density (as also pointed out for *A. arbustorum*, see Baminger and Haase 2000). Moreover, it should also be taken into account that population density may not be stable over time in the populations, which would result in fluctuating sperm competition intensity (as also argued by Baminger and Haase 2000).

One likely explanation for having longer sperm storage organs would be a higher sperm storage capacity (Pitnick

et al. 1999; Miller and Pitnick 2003). When attempting to explain the patterns of sperm utilization in *C. aspersum* snails, Rogers and Chase (2002) proposed a mechanism based on the activity of the allosperm in the spermatheca. The authors suggested that the unified beating of the flagella of the resident sperm would generate resistance to incoming sperm from subsequent mates (Rogers and Chase 2002), with this resistance becoming stronger when the sperm numbers are higher (Beese et al. 2006). In turn, this may have favored the increase in the length of spermathecal tubules (Beese et al. 2008), which would predict that more sperm can be stored, possibly also from more mating partners (see Baminger and Haase 1999; Pitnick et al. 1999; Miller and Pitnick 2003). Indeed, in land snails, sperm has been reported to be stored with their heads in tight contact with the epithelium of the spermathecal tubules (*A. arbustorum*: Bojat et al. 2001b; *C. aspersum*: Rogers and Chase 2002; *Bradybaena fruticum*: Bojat et al. 2002). Hence, longer spermathecal tubules may provide a larger surface area for sperm anchoring (Bojat et al. 2001b). In separate-sex species, as a result of an enhanced sperm storage capacity, females may benefit from a greater control over the fertilization process (Pitnick et al. 1999; Miller and Pitnick 2003) and possibly enhance cryptic female choice (Simmons 2001). In the previous studies, the only reported change was the volume expansion in the spermatheca of *A. arbustorum* snails occurring after sperm uptake. However, it was an expansion in the diameter of tubules and not their length (see Beese and Baur 2006). In our case, we did not consider the diameter of the tubules and we did not quantify the amount of sperm stored, since we sampled snails with an unknown mating history.

Ultimately, the actual effect of such complex spermathecae, and their variability, on paternity success remains to be demonstrated in land snail species (see also Baminger and Haase 1999). On one hand, considerable variation in sperm utilization patterns has been reported in investigations of double or triple mated snails (Murray 1964; Baur 1994; Evanno et al. 2005; Garefalaki et al. 2010). On the other hand, so far, increase in paternity success seems to be mostly linked to behavioural and some anatomical traits (Landolfa et al. 2001; Rogers and Chase 2002; Garefalaki et al. 2010) which act mainly on the number of sperm transferred and stored. This could suggest that the outcome of sperm competition may be determined by the proportional representation of sperm in the sperm-storage organ if sperm are randomly selected for use, as proposed by Landolfa et al. (2001), but this also remains to be demonstrated.

The inverse correlation that we found between shell size and population density confirms the findings on other snail species investigated either in the field or laboratory (Williamson et al. 1976; Oosterhoff 1977; Tattersfield 1981;

Baur 1988; Perry and Arthur 1991). Baur (1988) hypothesized that food unpalatability caused by mucus deposition slows down juvenile growth rate in high density populations, resulting in small adults and even reduced fecundity in subsequent years. In contrast, Baminger and Haase (1999) did not find any density dependence of shell breadth in their study on *A. arbustorum*, probably because of the influence of environmental factors. Similarly, Koemtzopoulos and Staikou (2007) did not find such a relation in their populations of *C. aspersum* from the highest humidity sites, which may partly confirm the interpretation of Baminger and Haase (1999).

The lengths of the reproductive organs considered in this study (dart sac, digitiform glands, epiphallus, flagellum, bursa copulatrix tract/bursa stalk, and diverticulum), which were expected to interact with the sperm-storage organs morphology (see "Materials and methods"), were not seen to vary significantly among the different populations investigated. Furthermore, they were not correlated with snail density. On the contrary, in *A. arbustorum,* the size of the same organs was found to be inversely related to local snail density in six natural populations (Baminger and Haase 2000). As mentioned above, these authors also attributed such a relation to the potential inhibitory effect of the presence of lots of snail mucus. As a consequence, they could not unambiguously determine the possible effect of sperm competition on the variation of these organs, which was the main goal of their study.

Given that we found no effect of shell size, this seems to indicate that the sperm storage organ is developmentally independent from body size in *H. aperta*, which is in agreement with most previous studies (Haase and Baur 1995; Baminger and Haase 1999, 2002; Bojat and Haase 2002; Koemtzopoulos and Staikou 2007; but see Beese et al. 2006). In addition, contrary to our expectations, no correlation was detected between any of the reproductive organs considered and the spermathecal structures of *H. aperta*. In contrast, phylogenetic study on the presence and complexity of the spermatheca in 47 Stylommatophora gastropods found that the complexity of this organ was associated with the occurrence of love darts and long flagella Beese et al. (2008). While this indicates an evolutionary association between these reproductive structures across species, we do not find this same relationship back when comparing within a single species.

Overall, our findings in *H. aperta* are suggestive of sexual selection acting on the structure of the spermatheca. Hence, these results contribute to a better understanding of the evolution of the diverse and complex sperm-storage organs seen in land snail species and even in other animal groups. Clearly, studies on these aspects in more organisms, with the inclusion of other factors such as behavioural traits as was done very recently in *C. aspersum*

(Garefalaki et al. 2017), can still further expand our understanding of sperm storage.

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