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MATING OF THE SLUGS *ARION LUSITANICUS* AUCT. NON MABILLE AND *A. RUFUS* (L.): DIFFERENT GENITALIA AND MATING BEHAVIOURS ARE INCOMPLETE BARRIERS TO INTERSPECIFIC SPERM EXCHANGE

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ABSTRACT

The large slug known as *Arion lusitanicus* (or *A. vulgaris*) is an important pest that is spreading through much of Europe. *Arion rufus* disappears at sites where *A. lusitanicus* has established strong populations. The finding of morphological intermediates suggests that *A. lusitanicus* hybridizes with *A. rufus*, but interspecific mating had not been proved. Considering the marked differences in their genitalia, it has been hard to envisage how mixed couples might transfer sperm. *Arion lusitanicus* and *A. rufus* were collected from pure populations near Görlitz, Germany, and used for laboratory mating trials involving either two individuals of *A. rufus* (henceforth RR), two of *A. lusitanicus* (LL) or one of each species (mixed). Matings were video recorded and some couples were killed during or after copulation to study spermatophore transfer and genital anatomy during mating. Three mixed pairs copulated. However, mixed pairs were significantly less likely to copulate than either RR or LL pairs (7% vs 52% and 36%). At each stage of mating, the probability of proceeding further was lower in mixed pairs than predicted from rates in RR and LL pairs, but this effect was strongest for yin-yang formation and initiating copulation. One problem was that *A. lusitanicus* tried to circle after yin-yang formation, whereas *A. rufus* remained stationary. In this respect, and in the repositioning of its everted oviduct, it was *A. lusitanicus* that compromised. LL copulations lasted over twice as long as RR copulations, but spermatophore formation took similar times, permitting reciprocal spermatophore exchange in mixed couples even though their copulations ended much earlier than in LL pairs. Our observations of mating behaviour of intraspecific pairs largely agree with previous descriptions of *A. rufus*, but we discuss some discrepancies between our findings and the fuller descriptions available for *A. lusitanicus*.

INTRODUCTION

Most terrestrial slugs have been described on the basis of their distal genitalia. If their genitalia differ, we assume that two taxa cannot successfully mate and thus deserve species rank under the biological species concept. One class of exceptions are species showing considerable intraspecific variation in their genitalia (e.g. Davies, 1977; Reise & Hutchinson, 2001). Another possibility is that two allopatric taxa differing consistently in their genitalia, and apparently good species, nevertheless manage to transfer sperm when artificially brought into contact. Wiwegweaw et al. (2009) provided an example in two snail species and Reise et al. (2011) observed other examples amongst *Deroceras* slugs. In these cases, sperm transfer was predominantly or only unidirectional, i.e. one species did not receive sperm. There are other examples of successful sperm transfer and even hybridization between well-defined gastropod taxa (e.g. Johnson, Murray & Clarke, 1993; Ribi & Oertli, 2000; Dillon, Wethington & Lydeard, 2011; Schilthuizen, Giesbers & Beukeboom, 2011).

We report here another example of interspecific sperm transfer, between the large slugs *Arion rufus* (Linnaeus, 1758) and *Arion lusitanicus* auct. non J. Mabille, 1868. *Arion rufus* is native in northwestern and possibly central Europe (Wiktor, 1973). *Arion lusitanicus* has spread through this area and parts of northern Europe over the last 60 years and continues to spread eastwards and northwards (Rudzīte et al., 2010; Engelke et al., 2011, and references therein). Its native range is usually assumed to be the Iberian Peninsula or southwestern France. Unfortunately, the original description of *A. lusitanicus* turned out to refer to a different species (Castillejo, 1998; Quinteiro et al., 2005), so the name *A. vulgaris* Moquin-Tandon, 1855 has been used as a replacement (Falkner, Ripken & Falkner, 2002). However, there remains uncertainty whether the original description of *A. vulgaris* really applies to the invasive species, so we follow Bank, Falkner & von Proschwitz (2007) in retaining the name *A. lusitanicus* until further clarification.

Arion lusitanicus is a much more damaging horticultural and agricultural pest than *A. rufus*, and occurs at noticeably higher densities (Schmid, 1970; Reischütz, 1984a; von Proschwitz, 1997; but see Davies, 1987, who described a more benign situation in Great Britain). We have monitored its spread in and around the town of Görlitz (on the Polish-German border) since the first records in 1994 (Reise, Backeljau & Seidel, 1996). At the very local scale, *A. lusitanicus* becomes common within a few years after arrival and *A. rufus* disappears (H. Reise, unpubl.). Reischütz (1984b), von Proschwitz (1997) and Kappes & Kobialka (2009) also reported the disappearance of *A. rufus* or its restriction to woodland at localities where *A. lusitanicus* reached high densities. Maybe *A. rufus* disappears because *A. lusitanicus* outcompetes or preys on it (von Proschwitz, 1997; Nordsieck, 2008). Another possibility is reproductive interference between the species through effort wasted in courting the wrong species (e.g. Hochkirch, Gröning & Bücker, 2007) and through further fitness costs if crossmating produces infertile eggs or sterile offspring. Indeed, the occurrence of hybrids is suggested by individuals with intermediate genitalia, which have been observed in the first years after the arrival of *A. lusitanicus* (H. Reise, unpubl.). Such intermediates have also been reported in Denmark (Engelke et al., 2011) and where *A. lusitanicus* has invaded the range of *A. ater*, a close relative of *A. rufus* (T. von Proschwitz, pers. comm.; Hagnell, Schander & von Proschwitz, 2003; see also Hatteland et al., 2010, for limited genetic evidence).

If these morphological intermediates are hybrids and can backcross with the parents, leading to introgression, then a further part of the reason for the disappearance of *A. rufus* might be swamping of its gene pool by *A. lusitanicus* genes (a widespread problem in other biological invasions: Rhymer & Simberloff, 1996; Huxel, 1999). Conversely, the invading *A. lusitanicus* may now be a hybrid that has picked up *A. rufus* genes as it spread across Europe. Heterosis might then contribute to its vigour (Hagnell et al., 2003; Arnold & Martin, 2010). But even without any selective advantage of *A. rufus* genes, even quite infrequent hybridization has the potential to lead to massive introgression of the resident's genes into the

invader's genome (Currat et al., 2008).

We examine in this paper whether the two species will mate and transfer sperm in the laboratory. This is a necessary, but of course not sufficient, step for the production of fertile hybrids. Were it not for the slugs with intermediate genitalia, successful interspecific crossing would have seemed unlikely, because the two species have such different distal genitalia. *Arion lusitanicus* has a long muscular distal oviduct containing a long fold, the ligula, while the atrium is small and not particularly muscular. The atrium and the distal oviduct are everted during copulation (Davies, 1987; Allgaier, 2000). The distal oviduct of *A. rufus* is much smaller and not particularly muscular, while its proximal atrium is large, swollen and thick walled and contains the ligula; only its atrium is everted during copulation (Simroth, 1885; Künkel, 1916; Gerhardt, 1940; Quick, 1947).

Since it turned out that the species can indeed transfer sperm interspecifically, this paper goes on to consider how they manage to do so, despite not only these morphological differences but also differences in mating behaviour. Although the mating behaviour of each species has been described before (*A. rufus*: Künkel, 1916; Gerhardt, 1940; Quick, 1947. *A. lusitanicus*: Davies, 1987; Allgaier, 2000; Kozłowski & Sionek, 2001; Sionek & Kozłowski, 2001; Gural-Sverlova & Gural, 2011), apparent species differences could be due to different conditions (e.g. temperature or substrate) or to different observers. Accordingly, in the Results section we describe afresh, and in greater detail, the mating behaviours of both species and of mixed pairs in a common laboratory setting. However, we will first summarize the mating process so as to define the successive stages involved (authors vary in how they apply these terms).

Courtship starts with an initiation phase in which one or both slugs nibble on the partner's body and then one closely follows the other's tail. If this becomes mutual, they form a circle, and such a configuration often precedes the next phase, which is the yin-yang configuration: the bodies lie bent, pointing in opposite directions, and thus hook round each other, bringing the genital openings into contact. During this phase, the genitalia may periodically partially evert, apparently trying to engage with each other. Courtship ends and copulation starts when the genitalia further evert, showing as a large whitish mass between their bodies (Fig. 1A). During copulation, spermatophores are produced and inserted into the partner's genitalia. Copulation ends when the slugs pull apart, each leaving its own spermatophore in the partner. Usually the tail of each spermatophore briefly remains sticking out, enabling us to recognize that transfer has occurred (Fig. 1B).

MATERIALS AND METHODS

Arion lusitanicus was collected in the Nikolai Cemetery, Görlitz, Germany (51.1602° N 14.9874° E) on 12 August 2010. The species had been known from here for about 10 years, and *Arion rufus* had disappeared by 2004. *Arion rufus* was collected 4 km away, from a locality at the margin of Zgorzelec, Poland (51.1458° N 15.035° E) on 7 August 2010. *Arion lusitanicus* had never been found here (nor was it the following year; the border and river delayed its invasion into the Polish side of the town, the earliest finding being 2006). Dissections of seven individuals of each species shortly after collection established that all had distal genitalia of adult size but the spermoviducts were not always fully grown. Comparing this with studies of wild populations (Smith, 1966; Grimm, 2001) led us to expect them to be already capable of mating or to become so during the course of the experiment. These dissections also indicated that neither population was mixed (confirmed after the experiment by dissecting the experimental animals).

Thirty-six individuals of each species were isolated 2 and 3 d after collection. They were kept in plastic containers (120 × 120 × 60 mm) containing several layers of damp tissue paper and fallen beech leaves. Food consisted of lettuce, carrot, oats and cat-food pellets. Containers

and contents were changed twice weekly. The containers were kept in temperature- and light-controlled chambers (17° C and 12L:12D) and in a cellar at 18–21° C with a natural day-night light regime.

Mating trials ran between 16 August and 15 September 2010. On each of 16 occasions, we set up 8–13 pairs simultaneously. Initial trials involved three pairs of *A. lusitanicus* (henceforth LL), three pairs of *A. rufus* (RR) and three mixed pairs, but we subsequently increased the proportion of mixed pairs. Slugs were isolated again when the mating trial ended and reused in later trials, but we avoided retesting individuals that had recently mated successfully; no trials are included where an individual mated <6 d before. (Two successful matings did occur 6 d after one partner had mated.) Individuals were paired randomly under the constraint that partners should be of roughly similar size. This resulted in some combinations of individuals being set up repeatedly. In our analysis of mating success, we considered the same combination multiple times only if a repetition was six or more days later (we judged it inappropriate to exclude all such repetitions, because motivation to mate evidently did change over this time scale). We also ignored the five sets of mating trials after 9 September because no pairs copulated after this date; potentially they had matured to the nonmating female stage (Smith, 1966). Twenty-four individuals laid eggs before this date, but some such individuals mated soon after laying or had done so shortly before, so we did not exclude trials involving these individuals (except once when eggs were laid during a trial itself). The analysis of mating success thus considers 23 RR trials (involving 23 individuals and 21 different combinations of individuals), 22 LL trials (23 individuals, 14 combinations) and 42 mixed trials (43 individuals, 30 combinations). Some combinations of individuals mated successfully only in the 99 further trials excluded under the above criteria. Data from these trials contributed to various other statistical analyses, for instance when comparing copulation durations; if the same pairing of partners had been set up repeatedly so that it generated several such durations, our analysis used a single median value for that pairing.

Each pair was put in a plastic box (115 × 150 × 90 mm) carpeted with wet tissue. Initially the pairs were observed directly under red light and their behaviours noted down. Once the yin-yang configuration formed, they were video recorded under low-level white lighting. We used an ISIS TFS-0406 digital video recording card allowing four-channel recording from Fujitsu CG-311 and Samsung SHC-737P cameras with Fujinon YV10x5B-2 lenses. Pairs not already mating were separated after 4 h. To study anatomy and spermatophore formation and transfer, four RR couples, four LL couples and one mixed couple were killed rapidly during copulation using boiling water. Two pairs of each species and one mixed pair were killed with carbonized water after the end of copulation to examine whether spermatophores had been exchanged. The dissections were drawn, initially using a camera lucida, from material preserved in 75% ethanol.

RESULTS

Failures and successes

Our most important result is that three mixed pairs copulated. However, the proportion of pairs proceeding to copulation was significantly lower in mixed pairs (7%; Table 1) than in both LL pairs (36%, $P = 0.006$, Fisher's exact test, two-tailed) and RR pairs (52%, $P < 0.0001$). To address the concern that this difference might be driven by a higher proportion of mixed pairs being set up at later dates, we repeated the analysis having discarded pairs (randomly when there was a choice) so as to ensure that on each day the number of mixed pairs matched that of the LL or RR pairs: the same pattern persisted (now $P = 0.02$ and 0.0002).

Was this infrequency of copulation in mixed pairs due to difficulties with a particular

stage? Consider first the proportion of pairs in which at least one partner showed any kind of interest in the other (e.g. nibbling). Mixed pairs less often showed interest than RR pairs (76% vs 96%), but the mixed and LL pairs were similar (76% vs 64%). Our null hypothesis is that whether a slug shows interest is independent of its partner's identity. Since either partner can show interest even if the other is uninterested, a proportion r_{RR} of RR pairs not showing interest indicates a probability of $r_{RR}^{0.5}$ that an *A. rufus* individual will be uninterested. Hence we predict

$$r_{mixed} = r_{RR}^{0.5} \times r_{LL}^{0.5} = (1 - 0.96)^{0.5} \times (1 - 0.64)^{0.5} = 0.13.$$

So, this null hypothesis predicts that 37 mixed pairs (87%) show interest, not much above the 32 observed. (Because the statistical error associated with our prediction is hard to estimate, we emphasize the effect size at each step rather than statistical significance; we have already demonstrated a significant difference overall.)

Of those pairs that showed some interest, the mixed pairs less often proceeded to form a yin-yang configuration than did LL and RR pairs (41% vs 64% and 77% respectively). Unlike with showing interest, we suppose that the yin-yang formation requires both partners to be willing. Under the null hypothesis of willingness being independent of the partner's identity, a proportion s_{RR} of RR pairs forming a yin-yang configuration indicates a probability of $s_{RR}^{0.5}$ of each partner being willing. Thus we derive the prediction that 23 ($70\% = 0.64^{0.5} \times 0.77^{0.5}$) of mixed pairs that showed interest should have formed a yin-yang configuration, approaching double the 13 observed.

Of those pairs forming a yin-yang configuration, the mixed pairs less often proceeded to copulation than did LL and RR pairs (23% vs 89% and 71%). Similar arguments as in the preceding paragraph predict that 10 mixed pairs (79% of those courting) should have proceeded to copulation, over three times as many as the three pairs that did so. To summarize, mixed pairs proceeded at each step less often than we predict from the behaviour of intraspecific pairs; the difference is substantial for forming a yin-yang configuration and starting to copulate, and little for showing initial interest.

All but five pairs of the 27 that copulated were either observed exchanging both spermatophores as they separated or found to be preparing both spermatophores when killed during copulation. The five exceptions were all RR pairs (out of 14 RR pairs that copulated). One of these exceptions is uninformative, involving an undissected couple not observed at the end of copulation. Three are cases where only one or no spermatophore was seen at separation, which is also uncertain evidence of failure because dissection of the animals involved in another such case showed that spermatophores had actually been exchanged. The fifth exception was a pair killed during copulation: dissection showed that only one partner had manufactured a spermatophore. Of the three mixed pairs that copulated, two were seen to successfully exchange spermatophores reciprocally (Fig. 1B; in one pair, this was further confirmed by dissection) and both partners of the mixed pair killed during copulation had formed a spermatophore.

Description and comparison of mating behaviours

We did not detect interspecific differences in the early phases of mating behaviour. Mating started when one partner, or sometimes both, nibbled the other. The nibbled slug usually crawled away, but the other followed, periodically nibbling the partner's tail. After some minutes, the leading slug doubled back. Often it then started nibbling the other's tail, and the couple rotated clockwise in a wheel-like configuration. This was only for a minute or two before gradually moving into the yin-yang configuration over several minutes. Other couples moved directly into the yin-yang configuration (i.e. no wheel configuration) when the leading

slug doubled back.

Even in pairs that eventually copulated, partners that had started interacting (e.g. one following the other) might break off contact with each other for some time before resuming courtship. Although in most cases no pause exceeded 15 min, sometimes yin-yang formation was preceded by one to five such pauses each of over 15 min, .

The yin-yang configuration was the position taken up for copulation, but it took some time until the atria everted fully (median 9 min, range 4–23 min; no difference among RR, LL and mixed pairs: $P = 0.8$, Kruskal–Wallis test). During this phase, it was impossible to see precisely how the genitalia interacted, because of the close contact between partners. In RR and mixed couples, small white genital structures appeared between the partners but were repeatedly retracted and re-everted (over a period of 4–14 min in those couples that then fully everted, longer in those that did not). As this happened, the anterior part of their bodies repeatedly twisted round to the right, apparently so as to bring the genital opening closer to that of the partner. LL couples also showed this twisting, but only in half the couples could we spot genital structures being partially everted and retracted. In both species, the structures repeatedly everting appear to be the part of the atrium carrying the openings of epiphallus and pedunculus (the duct leading to the bursa copulatrix). Particularly in couples that eventually failed to copulate, the repeated eversions and retractions gave the impression of probing for the right position for genital coupling. Probing continued until coupled genitalia rapidly everted further or the slugs eventually separated.

Of those that formed a yin-yang configuration but failed to copulate, the mixed pairs persisted significantly longer than RR pairs ($P = 0.01$, Mann–Whitney test; medians 45 and 30 min; Table 1). All but two of these 12 mixed pairs also persisted longer than the single LL pair in this category ($P = 0.3$). Their greater persistence suggests that it was not inadequate motivation that prevented mixed pairs from copulating. Nearly all pairs that failed to proceed persisted in the yin-yang configuration beyond the maximum time that successful couples required before starting to evert their atria fully (Table 1).

In both species, the full eversion of the atria at the start of copulation took only 1–2 min. Then, in *A. lusitanicus* the distal oviducts started to evert; oviduct eversion was much slower and less obvious than that of the atria. Each oviduct appeared first anterior to the atrium and, as it expanded, usually curved backwards onto the atrium. The expansion was often in pulses and usually not synchronous with that of the partner's oviduct. The point at which an oviduct reached full eversion could be hard to define, because some moved once expanded, or temporarily contracted, or lay partially hidden; expansion was often complete in 3–20 min, but could continue for over an hour.

The RR couples usually stopped rotating once they achieved the yin-yang position and remained static until the end of copulation; only exceptionally did some rotation occur, apparently to find a better position when the genitalia had not coupled properly. But all LL couples continued rotating (although slowly) when they achieved the yin-yang position, stopped for atrium eversion and then resumed rotating for another 4–66 min (median 27 min, $n = 6$). The rotation slowed over this period, becoming almost imperceptible before stopping. The remaining 194–230 min of copulation was motionless. This species difference (only LL rotate during yin-yang and copulation) caused problems in mixed pairs: the *A. lusitanicus* partner tried to continue rotating when the couple had attained the yin-yang configuration, but the *A. rufus* partner remained still. This could lead to the *A. lusitanicus* partner crawling onto the *A. rufus* partner. In the three mixed pairs that did copulate, eventually the *A. lusitanicus* partner had adapted by also remaining still. But in one of these pairs the *A. lusitanicus* partner resumed rotating after the atria had fully everted. Because its *A. rufus* partner did not rotate, the couple ended up parallel with the heads alongside each other, with the *A. lusitanicus* partner apparently stopped by the connected genitals. The couple remained in this unusual configuration and exchanged spermatophores successfully.

Once the genitals had fully everted, couples stayed in the same configuration for a long time (Table 1). However, in some LL couples, the position of the everted oviducts appeared less static than the atria of RR couples, sometimes uncovering the epiphallus-pedunculus complex. Three LL couples even partially retracted their oviducts and then everted them again, but all three successfully exchanged spermatophores. Besides this, once circling stopped, the only noticeable movements were waves of contraction across the everted atria or oviducts and occasional closing of the pneumostome.

Copulation lasted roughly three times as long in LL as in RR pairs, with no overlap in the ranges (medians 261 and 90 min: $P = 0.003$, Mann–Whitney test; Table 1). The two mixed pairs allowed to finish copulating took 82 and 145 min; the former is within the range of RR, the latter is 27 min longer than the slowest RR pair but 76 min shorter than the quickest LL pair.

Near the end of copulation, one partner would start moving its head, then become increasingly active and nibble the partner. The partners were rarely synchronized in starting to move, with a median difference of 3 min (range 8 s to 15 min; species similar). Then the atria or oviducts contracted, exposing the still-connected epiphallus-pedunculus complex. Within a few minutes, the partners started to pull apart, stretching out the epiphallus-pedunculus complex until this separated, usually exposing the tails of the spermatophores. This process (from the first 'wakening' movements until genital separation) was significantly faster in RR than in LL couples (medians 4 and 14 min, ranges 2–8 and 12–27 min: $P = 0.003$, Mann–Whitney test). The durations in the two surviving mixed couples were typical of those of LL couples. After separation each slug soon completed genital retraction (median 33 s). With the restart of activity, couples had recommenced rotating clockwise, continuing until full retraction or a little later (9–19 min of rotation, but the median number of revolutions was only 1.5). However, the two mixed couples did not rotate, presumably because the *A. lusitanicus* partner was unwilling to move so early (from its perspective); the *A. rufus* partner appeared to have to struggle to disengage its genitalia.

Mating anatomy

During the main part of copulation, mostly all that could be observed were two whitish balloon-like structures filling out the space between the partners and apparently pressed against each other (Fig. 1A; Fechter & Falkner, 1990: p. 195). These are the atria in *A. rufus* (Fig. 2) and the distal oviducts in *A. lusitanicus* (Fig. 3). Beneath these structures, mostly hidden, were the genital parts involved in the exchange of spermatophores; the opening of the epiphallus lay just anterior to that of the pedunculus (Figs 2, 3).

In copulating *A. rufus*, the pedunculus and the epiphallus were slightly everted, roughly to equal extents, and sat on a common base somewhat elevated from the atrium (Fig. 2). From the opening of the epiphallus a papilla-like structure stuck out prominently. The pedunculus opening appeared merely to press against the epiphallus opening, involving hardly any insertion except of the papilla into the pedunculus (Simroth, 1885; Künkel, 1916). There is a muscular ring structure around the epiphallus opening (called the "vestigial phallus collar" by Barker, 1999), which might grip the slightly everted pedunculus. However, any such hold was insufficient to prevent all four couples killed during copulation from falling apart (despite the partially transferred spermatophores linking them).

In copulating *A. lusitanicus*, the base of the pedunculus was everted further than in *A. rufus* and it protruded, chimney-like, much further than did the epiphallus (Fig. 3C). The two structures lay close together, but were not elevated on a common base. There was a papilla-like structure, but it was weaker and hidden inside the epiphallus opening. Each slug's everted pedunculus was inserted into the partner's epiphallus (probably up to the insertion of the papilla) and was probably gripped by the ring around the epiphallus opening. The connection

may be firmer than in RR couples, since some LL couples killed with boiling water remained coupled (even in the absence of partially transferred spermatophores linking them).

In LL couples, the everted oviduct of each partner usually lay dorsal and anterior to the rest of the everted genitalia (Figs 3, 4C–D), having the shape of a bent and swollen sausage. The side of the everted oviduct that carries the ligula was directed towards the epiphallus-pedunculus complex. Although the two oviducts typically hid the epiphallus-pedunculus complex from above and from the side, often, and in some couples most of the time, a part or all became visible from above between the two oviducts. Rarely, one of the oviducts remained anterior.

In RR couples, the swollen part of the everted atrium was larger and more nearly hemispherical than the oviduct of LL couples and also differed in inserting posterior to the epiphallus-pedunculus complex, curving round it over the dorsal, posterior and ventral sides (Fig. 2). Hence the species are distinguishable at a glance when they copulate. At the beginning of copulation, the atria in RR couples were positioned roughly symmetrically. Later they sometimes turned so that one atrium was more above and the other more below the genital mass. The atria of both partners together appeared to wrap the epiphallus-pedunculus complex completely. The part of the atrium directly contacting the epiphallus-pedunculus complex was the ligula. Between the ligula folds is a prominent structure formed by the entrance of the oviduct (Fig. 2E).

In mixed couples, the everted oviduct of the *A. lusitanicus* partner and the atrium of the *A. rufus* partner got in each other's way: according to each animal's own orientation, the former is anterior and the latter is posterior to the epiphallus-pedunculus complex, but the two orientations are antiparallel, hence the interference. The oviduct of *A. lusitanicus* was the part that 'gave in'. In the mixed couple killed during copulation (Fig. 5A–C), the oviduct of *A. lusitanicus* was less expanded than in LL couples and partly pressed downwards. In the two other mixed couples, the oviduct lay on top of the partner's atrium.

Spermatophore formation and transfer

Spermatophores of *A. lusitanicus* had a curved length of 20–34 mm ($n = 4$) with a large and strongly pointed denticulation along a longitudinal ridge (Fig. 6D). Spermatophores of *A. rufus* were similar in length (20–29 mm; $n = 4$), but thicker, and the ridge denticulation was less pointed (Fig. 6B).

In the two RR couples killed 29 and 34 min after copulation had started, unfinished spermatophores were found in the epiphalluses, partly sticking into the partner's pedunculus (Fig. 4A, B). They were transferred roughly halfway, but this is approximate because they were very misshapen, probably an artefact of the killing or preservation method. The two couples killed after 75 and 80 min contained apparently completed spermatophores, not misshapen, with still only one-third or half their length inserted into the partner's pedunculus (Fig. 2A–C).

The three LL couples killed 28, 53 and 58 min after the start of copulation contained spermatophores that were completely, or at least three-quarters, still in the epiphalluses. None were misshapen (presumably they had already hardened), although their tails were still unfinished (thicker than normal and spongy). Both partners in the couple killed after 108 min contained a completed spermatophore, of which about one-third was in the recipient's pedunculus (Fig. 4C, D).

In the mixed couple killed 64 min after the start of copulation (Fig. 5), the tails of both spermatophores were not yet ready. The spermatophore of the *A. lusitanicus* partner still lay entirely in its epiphallus, but one-third of the spermatophore of the *A. rufus* partner was already transferred.

To summarize, a time for spermatophore completion of about 70 min would be compatible

with all our data from both species as well as with Sionek & Kozłowski's (2001) study of *A. lusitanicus*. Seventy minutes is most of the copulation in *A. rufus* and mixed couples, but less than half the copulation in *A. lusitanicus*. Transfer into the partner started earlier in *A. rufus*, when the spermatophores were less complete.

In slugs killed shortly after copulation ended, the spermatophores extended from the atrium into the pedunculus in the one RR pair (Fig. 6A) and from the oviduct through the atrium into the pedunculus in the two LL pairs (Fig. 6C; in agreement with Sionek & Kozłowski, 2001). In the mixed pair killed at this stage, both spermatophores were in the atrium and pedunculus. In the RR pair killed 1 h after mating, the entire spermatophore was in the bursa copulatrix (roughly in agreement with Künkel, 1916).

Spermatophores from all couples contained some white ejaculate. For the critical examples of the mixed couple killed after copulation and the pairs of each species killed earliest during copulation, we confirmed microscopically that the ejaculate included sperm.

DISCUSSION

We have demonstrated reciprocal transfers of spermatophores between the species. The success rate in mixed pairs was only 16% of that in intraspecific pairs, but this would not by itself prevent massive introgression (Currat et al., 2008).

Our laboratory experiments in small containers with no choice of partner may conceivably overestimate success rates of mixed mating in the wild. But what also matters is how often an individual encounters the other species. On first colonizing a site, *A. lusitanicus* individuals are more likely to encounter *A. rufus* than their own species. This is particularly the case if, as we suspect, colonization by *A. lusitanicus* often involves passive transport with horticultural plants. Such a colony may involve few individuals, which are only several generations later likely to be reinforced by self-propelled immigration from nearby populations of *A. lusitanicus*. This probable restriction of choice early in the invasion process is one reason why we did not allow our slugs a choice of partner in our experiments. There are other advantages of no-choice trials (e.g. Rutstein, Brazill-Boast & Griffith, 2007) and anyway in nature potential mates will be encountered more often sequentially than simultaneously.

Although our experiments have demonstrated an opportunity for hybridization, we emphasize that direct proof that it occurs is lacking. Studies on other taxa have emphasized the importance of multiple isolating barriers in preventing hybridization and introgression (Sanchez-Guillen, Wullenreuther & Cordero Rivera, 2011). Slugs in our experiments mated multiple times (in both species some individuals mated three times) and both species can self (Künkel, 1916; Hagnell, von Proschwitz & Schander, 2006), so hybridization would be hindered if intraspecific sperm compete better to fertilize eggs. We have confirmed neither whether cross-species fertilization produces viable embryos, nor whether these correspond to the morphological intermediates observed in the wild. Even if hybrids form, the F1 generation could be sterile or unable to backcross.

The willingness of mixed pairs to court matches our observations of several species of Deroceras slugs that also will persistently court the wrong species, at least in captivity (Hutchinson & Reise, 2009; Reise et al., 2011). More surprising to us was that the two *Arion* species overcame considerable differences in the morphology of their genitalia, in which parts of their genitalia they evert, and in mating behaviour (duration of copulation, rotating or not). In other pulmonates, differences in timing create a barrier to hybridization at least in one direction: the species that normally takes longer to transfer sperm fails to act in the male role (Wiwegweaw et al., 2009; Reise et al., 2011). But *A. lusitanicus* was able to donate a filled spermatophore to *A. rufus* in half the time it took to complete copulation in an LL mating.

Part of the explanation is that in LL matings the spermatophore is already completed and much of it is transferred halfway through copulation (our data and Sionek & Kozłowski,

2001). This prompts the question why LL matings continue for the extra 2 h. There must surely be predation and desiccation risks in sitting exposed for the extra time; possible compensatory benefits include packing more ejaculate in the spermatophore, assessment of partner quality or partner manipulation.

In other respects also, it is mostly the *A. lusitanicus* partner that adapts where interspecific differences might inhibit sperm exchange: besides shortening its copulation, it suppresses circling during late courtship and early copulation, and repositions its oviduct. This might reflect the recent history of invasive *A. lusitanicus*: in the initial stages of colonization, there may be little opportunity for *A. lusitanicus* to mate intraspecifically, so, if it forms fertile hybrids, its adaptability in mating could have both been a preadaptation for invasiveness and been selected during the invasion process.

. We note in passing a report of cross-species spermatophore transfer from *A. distinctus* to *A. hortensis* (Iglesias & Speiser, 2001). The genital morphology and mating behaviour of these species are more similar than in our species (Davies, 1977), which may make hybridization both easier to occur and harder to recognize.

Differences between studies in reported mating behaviour

Our observations of the mating behaviour of *A. rufus* agree with Gerhardt's (1940) and Quick's (1947) descriptions (based on far fewer observations than ours). Our only disagreement with the extensive study of Künkel (1916) is his statement that the spermatophores were completed within the first 20 min of copulation. In our RR couples killed after 29 and 34 min, the spermatophores were distorted, indicating that their walls had not yet hardened. This might well not have been apparent to Künkel if he used different methods of killing or preservation.

Other descriptions of the mating of *A. rufus* give different durations of some mating phases. Bouchard-Chantereaux (1839) reported 2 h of head-to-tail following (copied by Moquin-Tandon, 1855, and Lams, 1910) and 30–40 min of yin-yang configuration before genital eversion. Jourdain (1878) reported 1–2 h of following. Plausibly, the initial phases of mating, particularly following, take longer in the field than in our containers; besides the uneven substrate and lower temperatures, there is also the ambiguity of how to score durations when the partners separate for a period, and the consequences of separation are likely to be different in containers than in the field. More surprising are discrepancies in the duration of copulation. Several early studies (Moquin-Tandon, 1855; Jourdain, 1878; Lams, 1910) reported 1 h, compared with our observed range of 88–127 min. However, it is unclear how many copulations these observers timed, Moquin-Tandon's and Lams's reports were certainly conflated with observations on *Limax* and, since Jourdain and Moquin-Tandon mentioned no locality, they could have been dealing with an *Arion* species other than *A. rufus*. Chevallier (1974) distinguished several subspecies of *A. rufus* in France and reported copulations taking under 80 min for one, over 110 min for another and over 240 min for a third; however, it reads as if these figures might derive from single observations. Reports of much shorter copulations in Britain likely all refer to *A. ater* (Wotton, 1893; Adams, 1910; Gerhardt, 1940; Quick, 1947).

For *A. lusitanicus*, particularly thorough observations of mating have been made by Allgaier (2000), based on field observations in Baden-Württemberg, and by Kozłowski & Sionek (2001) and Sionek & Kozłowski (2001), based on field observations in southeastern Poland. For the initial phase before yin-yang formation, Kozłowski & Sionek (2001) reported a duration of 10–24 min and Allgaier wrote "rarely less than 30 min", compared with our figures of 12–168 min. As with *A. rufus*, discussed above, we believe that these differences could arise from different conditions in the laboratory and the field.

Uneven substrates and lower temperatures in the field might also prolong the yin-yang

configuration prior to full atrium eversion: Allgaier (2000) reported 17–60 min while we observed only 5–18 min. For the full eversion of atria when genitals become clearly visible and push the partners apart, Allgaier (2000) reported 30 s followed by 10 min eversion of the oviducts. Our data largely agree, except that we observed oviduct eversion to vary considerably in duration (5 min to over 1 h). Kozłowski & Sionek (2001) were less specific in describing genital coupling and eversion, hindering comparison between studies. They reported 2–3 min for yin-yang formation and 9–24 min for the eversion of "atria and adjacent parts". We suspect the latter phase included the period in the yin-yang configuration, when partially everted genitalia can sometimes be glimpsed. In this case, its duration agrees quite well with our data (5–18 min plus 1–2 min for the full atrium eversion). Kozłowski & Sionek (2001) apparently did not observe the longer process of oviduct eversion.

Our LL copulations lasted 221–268 min, in agreement with the 190–255 min reported by Kozłowski & Sionek (2001), the 240–360 min reported by Allgaier (2000) and a single observation of 215 min from Scotland (Davies, 1987), but disagreeing with figures of 140 min from France (Chevallier, 1974) and 140–150 min from London (Davies, 1987). This London population was studied in the first season after *A. lusitanicus* was discovered at that site (Davies, 1987), so possibly *A. rufus* and hybrids were co-occurring. Our mixed couples copulated for similarly short times (82 and 145 min).

Allgaier (2000) reported the retraction process lasting 1–2 h, much longer than the retractions that we observed (12–27 min). Our figures agree with the 15–30 min reported by Kozłowski & Sionek (2001) and slightly different interpretations of the first retraction movements would also make this compatible with the 5–10 min reported by Davies (1987).

There is some disagreement between studies concerning when LL couples rotate. We observed rotation in the yin-yang stage before clearly visible genital eversion, then a brief stop just prior to full eversion of the atria, and then restarting of rotation, persisting for 4–66 min of copulation. Both Allgaier (2000) and Kozłowski & Sionek (2001) reported that rotation stopped once the genital pores were in close contact, thus before the atria started full eversion. According to Kozłowski & Sionek (2001), the couples remained motionless until the end of copulation. In contrast, since Allgaier (2000) mentioned that rotation almost stopped when the oviducts were fully everted (only roughly true in our study), his slugs must have restarted at some stage. Davies (1987) observed several phases of rotating during copulation in one couple from Glasgow, but none in couples from London (again explicable if these were mixed-species pairs). In part, the disagreements may stem from methodology; our video recordings make it easier to recognize and time rotation than when taking notes of behaviour during direct observation.

Our anatomical study (Figs 3, 4) demonstrated that the oviduct of *A. lusitanicus* is everted, in agreement with other authors (Davies, 1987; Noble, 1992; Allgaier, 2000; Gural-Sverlova & Gural, 2011). Kozłowski & Sionek (2001) seem to have disagreed, but there is some ambiguity: they mentioned "convexities of everted oviduct margins" being visible in some pairs. This seems to be a matter of interpretation rather than a population difference, because Figure 1 of Sionek & Kozłowski (2001) shows prominently everted oviducts.

Sionek & Kozłowski's (2001) study conflicts with ours in some aspects of the timing of spermatophore formation and transfer in *A. lusitanicus*. For instance, we found incomplete but well-formed spermatophores in a pair killed 28 min into copulation, whereas they found only an "amorphous substance" at that time. Couples that we killed 53 and 58 min into copulation had transferred little or none of the spermatophore to the partner, whereas Sionek & Kozłowski's (2001) found that already after 40–45 min the spermatophore was three-quarters transferred. [Confusingly, the same authors claimed later (Kozłowski & Sionek, 2001) that insertion into the pedunculus occurs at the end of copulation, which is hours later; this must be an error. We found appreciable transfer after 108 min.] This calls into question our conclusion that transfer starts earlier in *A. rufus* than in *A. lusitanicus*. Evidently there is more

variation in this process than suggested by the results of either study alone; different temperatures in field and laboratory may contribute. Nevertheless, both studies agree that in *A. lusitanicus* the spermatophores appeared complete and were substantially transferred well before the halfway point of copulation.

To summarize, the reliable descriptions of *A. rufus* mating are largely consistent, while the more detailed studies of *A. lusitanicus* show some discrepancies in durations and in when the slugs circle. Discrepancies might partly reflect methodological differences or different definitions of stages, but perhaps also differences between populations or even cryptic taxa. Population differences of a rapidly spreading species could arise from different local histories of introgression with *A. rufus*. It would be worthwhile for a single group of researchers to compare populations using a common protocol for scoring behaviours, ideally in a common laboratory environment.

Further aspects of functional morphology

Even though the everted genitalia are in part very different in *A. lusitanicus* and *A. rufus*, cross-species spermatophore transfer is possible because the structures most directly involved, the epiphallus and pedunculus, are sufficiently similar. In both species, the openings of these ducts lie close together, with that of the epiphallus anterior, and the ring structure around the epiphallus opening is well developed and muscular. The crucial process of coupling the genitalia during the yin-yang phase is hard to observe but the species behaved mostly similarly. In both, the partially everted genitalia could be glimpsed probing (although more prominently and consistently in *A. rufus*), and the anterior part of the bodies twisted backwards, apparently to facilitate the genital contact. Moreover, there was no species difference in the time required before the atria fully everted. But we identified a behavioural difference that did present problems for mixed pairings: only LL couples rotated during the probing phase.

The large everted oviduct of *A. lusitanicus* also required an awkward-looking compromise from mixed pairs. But since the oviduct everts only once the genitalia have coupled and the atria expanded, and since none of the three mixed couples that had got that far gave up at that point, it seems that this most prominent anatomical difference between the species is itself no barrier to cross-mating.

The structure of the epiphallus, the funnel-like everted pedunculus and the way that these interact in *A. lusitanicus* are also reminiscent of Webb's (1950) description of mating *A. subfuscus*. At rest, the distal genitalia of *A. lusitanicus* and *A. subfuscus* are very similar, so it is unsurprising that they are used similarly, even though these species are more distantly related than *A. lusitanicus* is to *A. rufus* (Quinteiro et al., 2005).

As the spermatophore is transferred counter to its dentition, the dentition cannot have evolved to enable the donor to grip the recipient, nor to facilitate spermatophore transfer and uptake by the recipient. [The latter was proposed by Kozłowski & Sionek (2001) and Sionek & Kozłowski (2001); although their illustrations show the dentition to be directed against the direction of transfer, they mistakenly interpreted the opposite.] According to Davies (1987), the spermatophores are covered by a thick layer of mucus, making the transfer easy. She considered a possible function of the dentition to act as a holdfast during spermatophore filling. Künkel (1916) suggested that the dentition gripping the recipient's genital wall serves to exert tension that splits the spermatophore open, so allowing the sperm to leave. We propose an alternative hypothesis: after spermatophore exchange, the dentition is orientated to hinder transport further along the pedunculus into the bursa copulatrix, thus helping the sperm to escape digestion. The opposite, preventing the spermatophore from being ejected from the bursa when 'exploding', was suggested by Simroth (1885) because he also got the direction of dentition wrong.

Noble (1992) states that the ligula is rubbed along the side of the partner and later grips the partner's ligula (in *A. rufus*) or both ligula and body (in *A. lusitanicus*); he concluded that it is an organ of both adhesion (see also Tompa, 1984) and stimulation, permitting the coupling of male and female openings. However, our observations show that the ligula everts only after genital coupling. In both species we did not observe the ligula rubbing along the partner's side [and not over its back, as indicated in Davies's (1987) figure of an *A. lusitanicus* couple]. Since the ligula faces towards the epiphallus-pedunculus complex, it also seems impossible that it could grip the partner's ligula (see also Allgaier, 2000). The ligula's function remains an open question.

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Table 1. Success rates and durations of components of mating behaviour in couples of *A. rufus* (RR), couples of *A. lusitanicus* (LL), and mixed couples.

	Species combination		
	RR	Mixed	LL
Numbers of couples at each stage, (%age of those set up), [%age of those at previous stage].			
Set up	23	42	22
Some kind of interest	22 (96%)	32 (76%)	14 (64%)
Yin-yang formation	17 (74%) [77%]	13 (31%) [41%]	9 (41%) [64%]
Copulation	12 (52%) [71%]	3 (7%) [23%]	8 (36%) [89%]
Median duration and range of each phase in minutes			
Courtship bout prior to yin-yang[1]	35 (13–133)	26 (12–75)	20 (12–168)
Yin-yang prior to copulation	9 (7–23)	7 (4–23)	10(5–18)
Yin-yang if not copulate	30 (24–36)	45 (21–107)	35
Copulation	90 (75–117)	82, 145	261 (221–268)

To avoid pseudoreplication, these figures exclude certain trials, as described in the Methods section.

[1]Note: Figures here describe only those courtship bouts leading up to a yin-yang and copulation. We define a new courtship bout as starting when there has been no interaction for > 15 min.

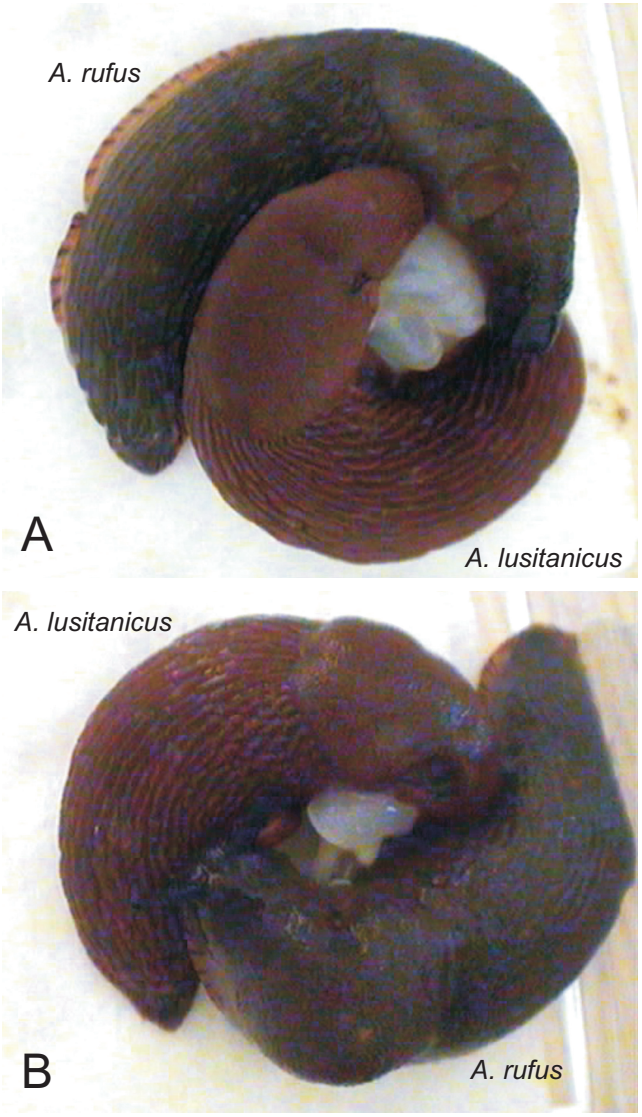

Figure 1. Two stages of copulation in a mixed couple. **A**. The middle of copulation. The everted atrium of *A. rufus* is almost completely hidden under the oviduct and atrium of *A. lusitanicus*. **B**. The end of copulation, when the two spermatophores can be seen connecting the retracting genitalia.

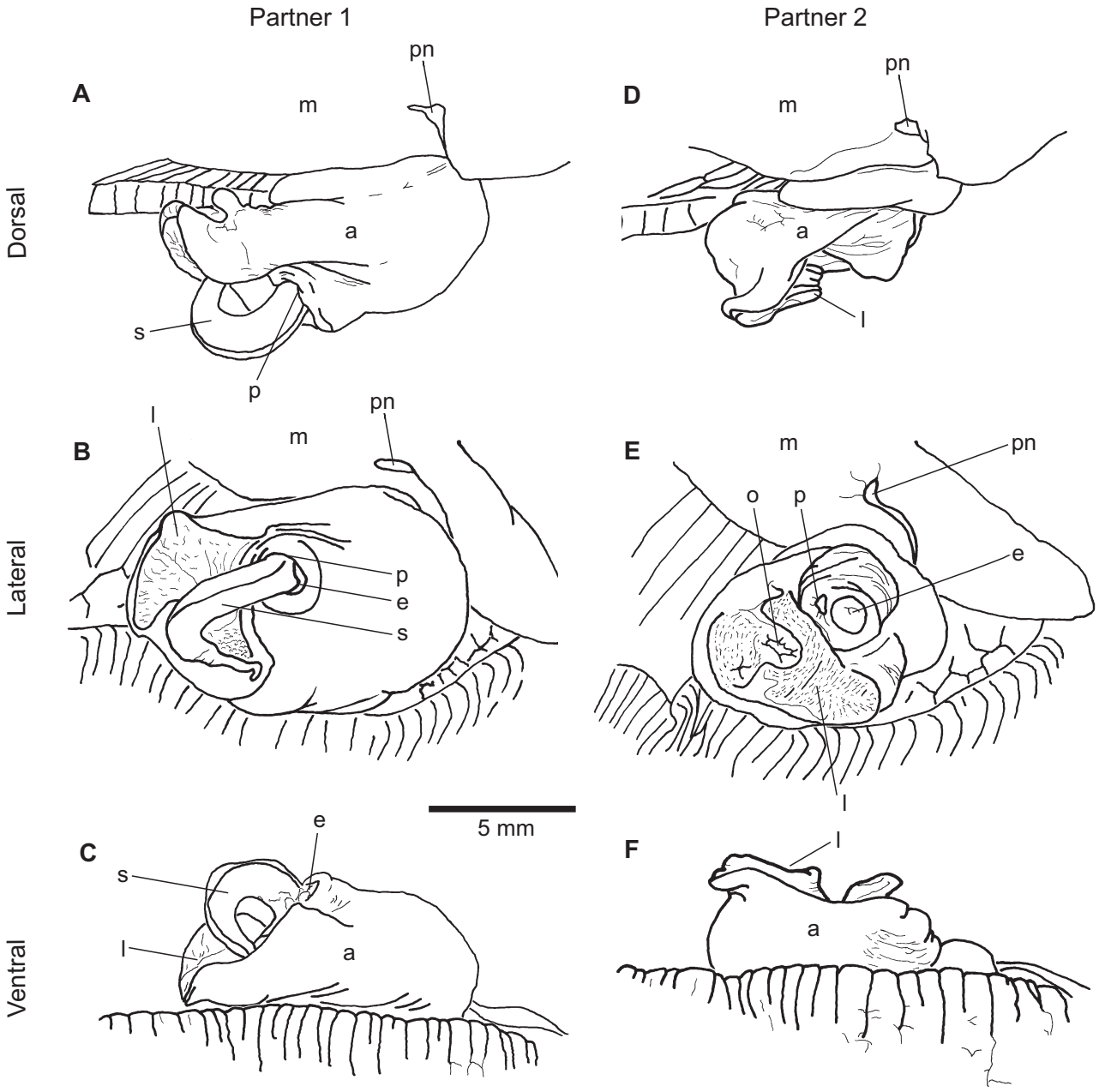

Figure 2. The everted genitalia of one couple of *Arion rufus* killed during copulation, 80 min after its start. Note that the position of bursa and epiphallus openings are similar as in *A. lusitanicus* (Fig. 3), but that the ligula with the oviduct opening is posterior to them and the oviduct is not everted (only the opening is visible). In the pedunculus opening of partner 1, a received spermatophore is visible. The opening of the epiphallus of partner 1 is unusually small and not visible, probably because, unusually, this partner did not produce a spermatophore. The atria have shrunk during the fixation process. Abbreviations: a, atrium; e, opening of epiphallus; l, ligula; m, mantle; o, opening of oviduct; p, opening of pedunculus; pn, pneumostome; s, spermatophore.

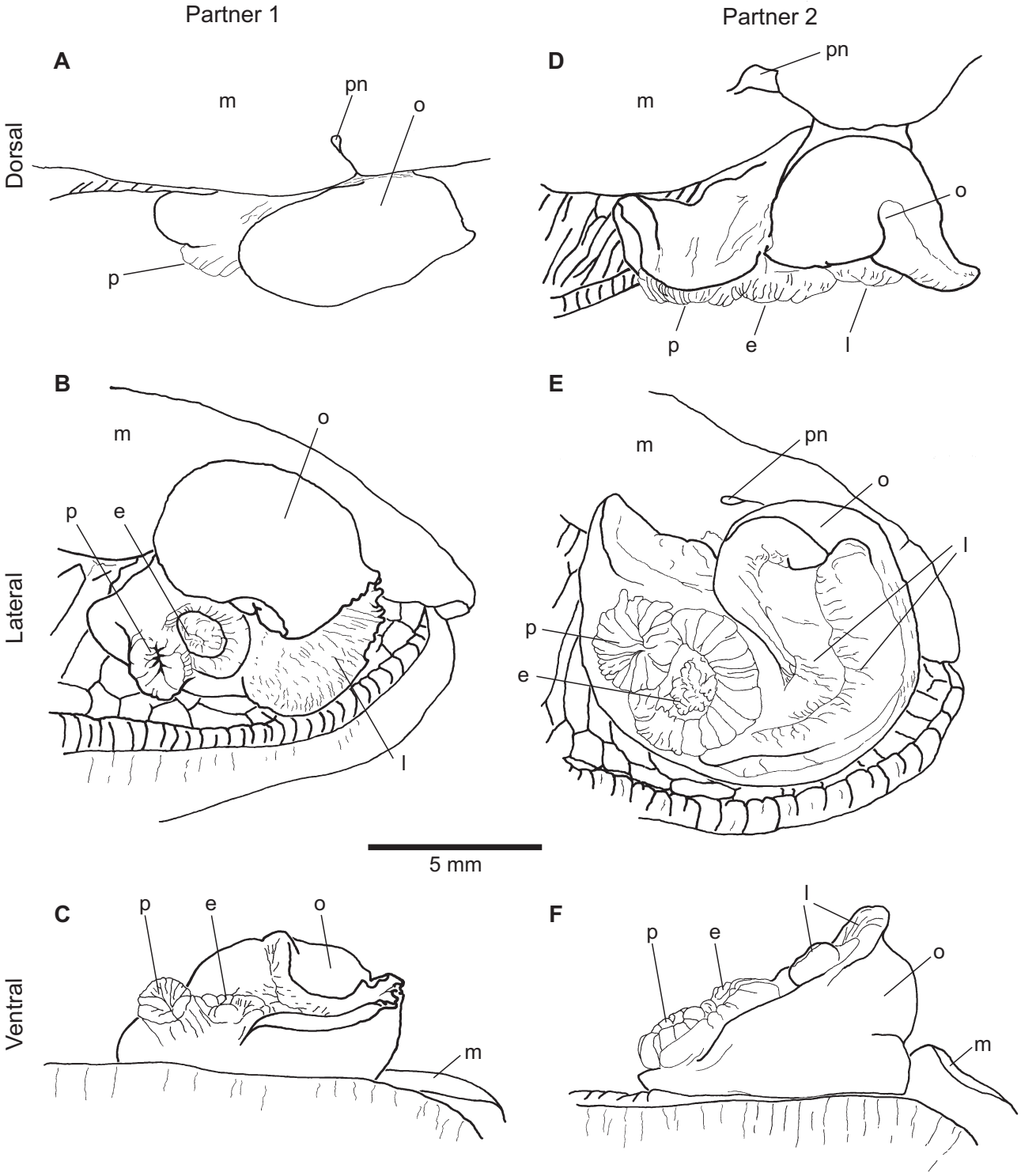

Figure 3. The everted genitalia of one couple of *A. lusitanicus* killed during copulation, 58 min after its start. The oviducts have shrunk during the fixation process, but note their anterior position. Abbreviations: e, opening of epiphallus; l, ligula; m, mantle; o, oviduct; p, opening of pedunculus; pn, pneumostome.

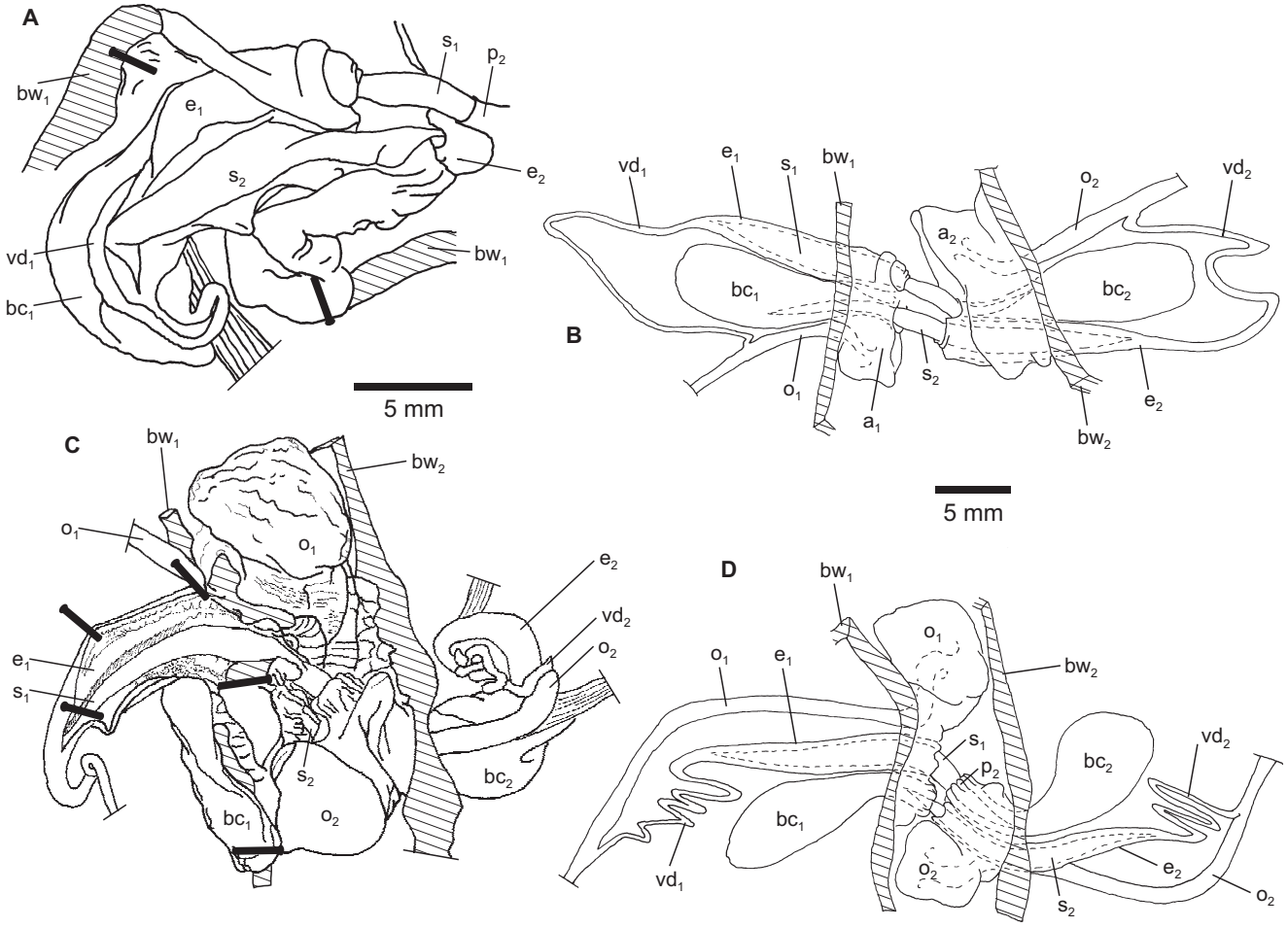

Figure 5. Mixed couple killed during copulation, 64 min after its start. The everted oviduct of *Arion lusitanicus* is squashed much flatter by the atrium of *A. rufus* than in LL couples (Fig. 3). A spermatophore sticks out from the epiphallus of *A. rufus*; this was in the partner's pedunculus. The spermatophore of *A. lusitanicus* is not visible, but was found in its epiphallus after dissection. Abbreviations: a, atrium; e, opening of epiphallus; l, ligula; m, mantle; o, oviduct; p, opening of pedunculus; pn, pneumostome; s, spermatophore.

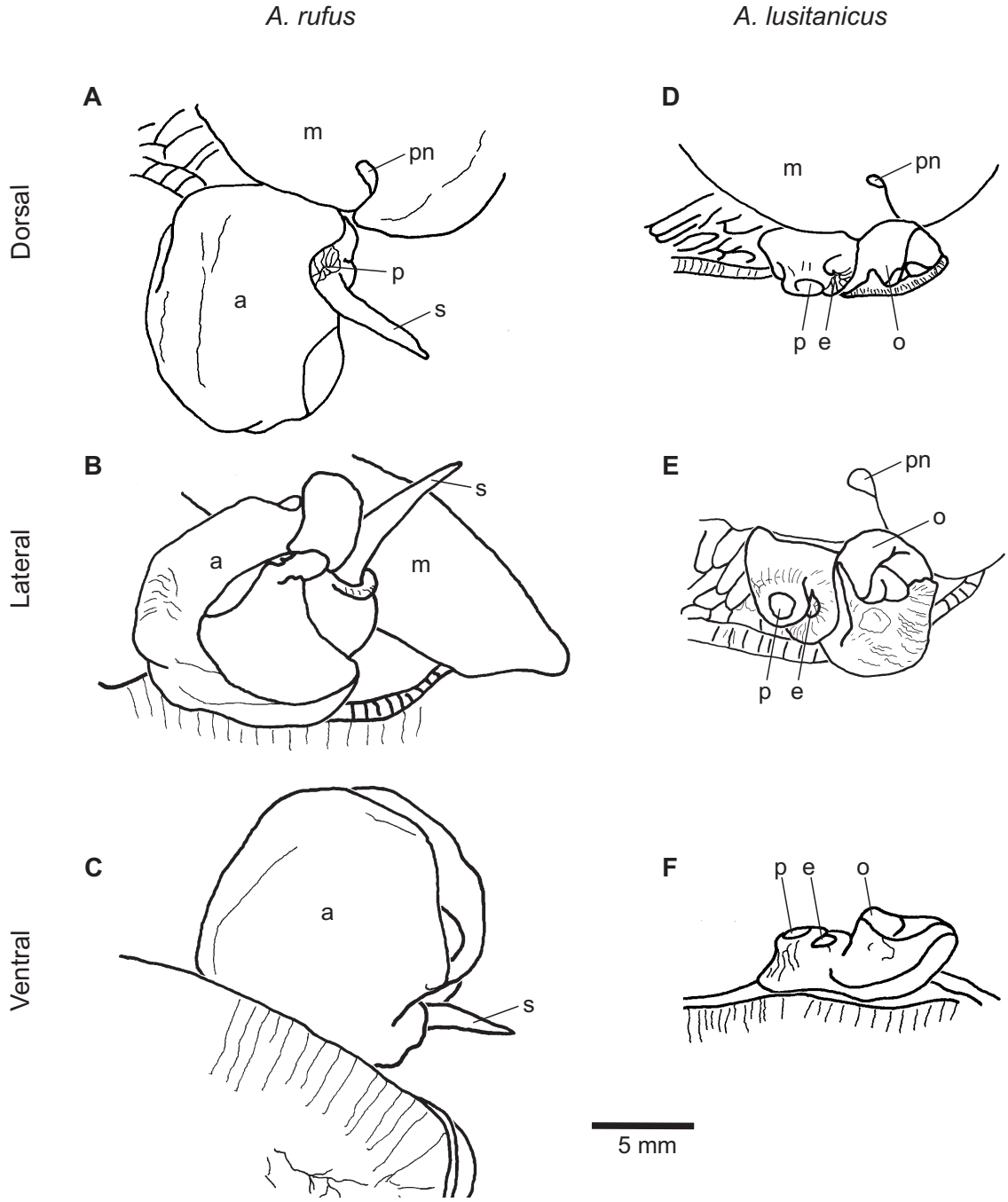

Figure 4. The anatomy of mating in *Arion rufus* (**A**, **B**) and *A. lusitanicus* (**C**, **D**), killed, respectively, 29 and 108 min after the start of copulation. The partners' genitals have separated slightly during fixation. **A**, **C**. Dorsal views of dissected alcohol-preserved specimens, which established the position and interaction of genitals and the spermatophores' position partly in the donor's epiphallus and partly in the recipient's pedunculus. **B**, **D**. Schematic versions of **A** and **C**, respectively. Broken lines indicate position of parts which are hidden from view. Hatching indicates where the body wall has been sectioned. Abbreviations: a, atrium; bc, bursa copulatrix; bw, body wall; e, epiphallus; o, oviduct; p, pedunculus; s, spermatophore; vd, vas deferens. Suffix (1 or 2) indicates partner.

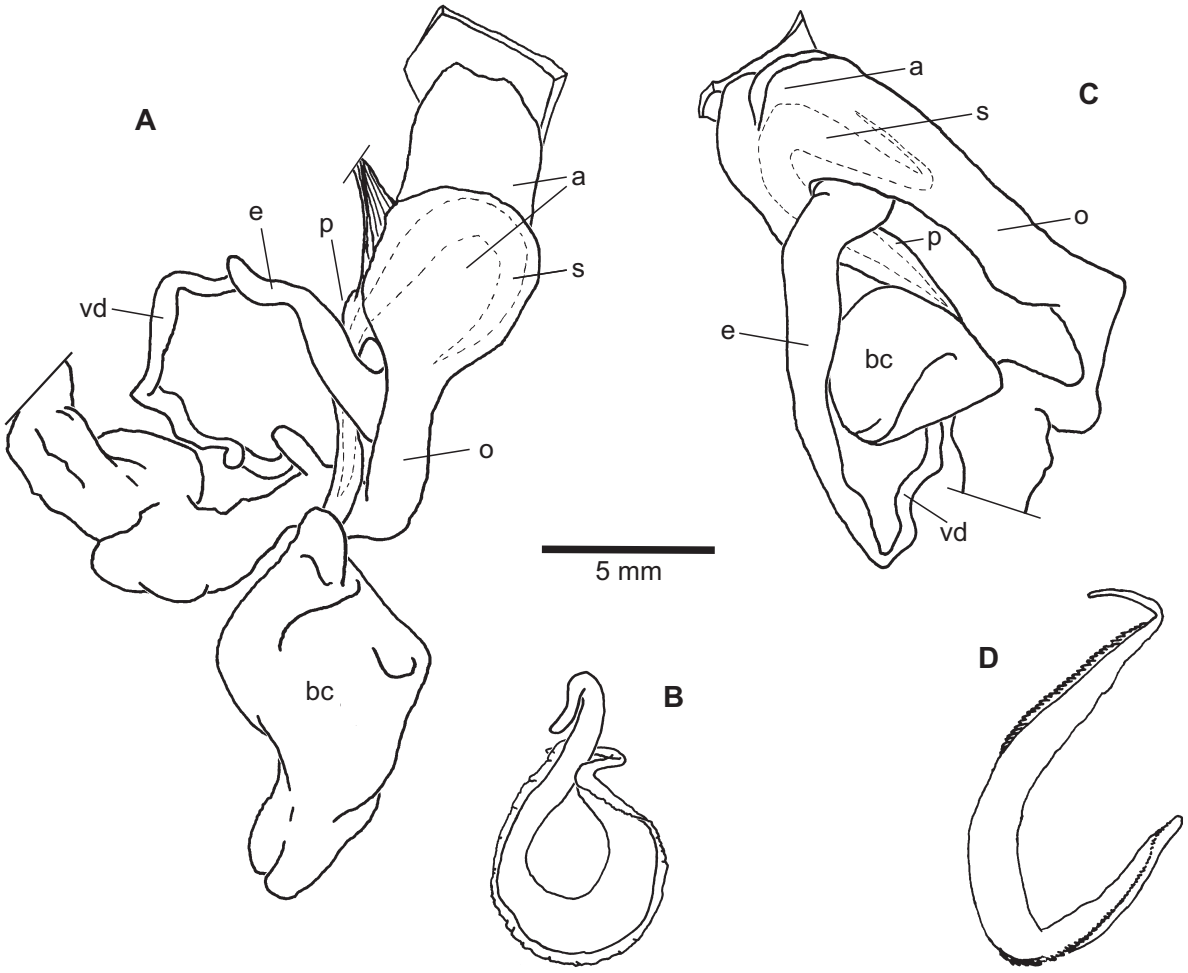

Figure 6. Distal genitalia (**A**, **C**) and spermatophores (**B**, **D**) of *Arion rufus* (**A**, **B**) and *A. lusitanicus* (**C**, **D**) killed shortly after mating. Broken lines indicates positions of spermatophores in the pedunculus, atrium and oviduct. Abbreviations: a, atrium; bc, bursa copulatrix; e, epiphallus; o, oviduct; s, spermatophore; vd, vas deferens.