

# SOME NOTES ON REPRODUCTIVE BIOLOGY AND MATING BEHAVIOUR OF *ARION VULGARIS* MOQUIN-TANDON 1855 IN NORWAY INCLUDING A MATING EXPERIMENT WITH A HYBRID OF *ARION RUFUS* (LINNAEUS 1758) X *ATER* (LINNAEUS 1758)

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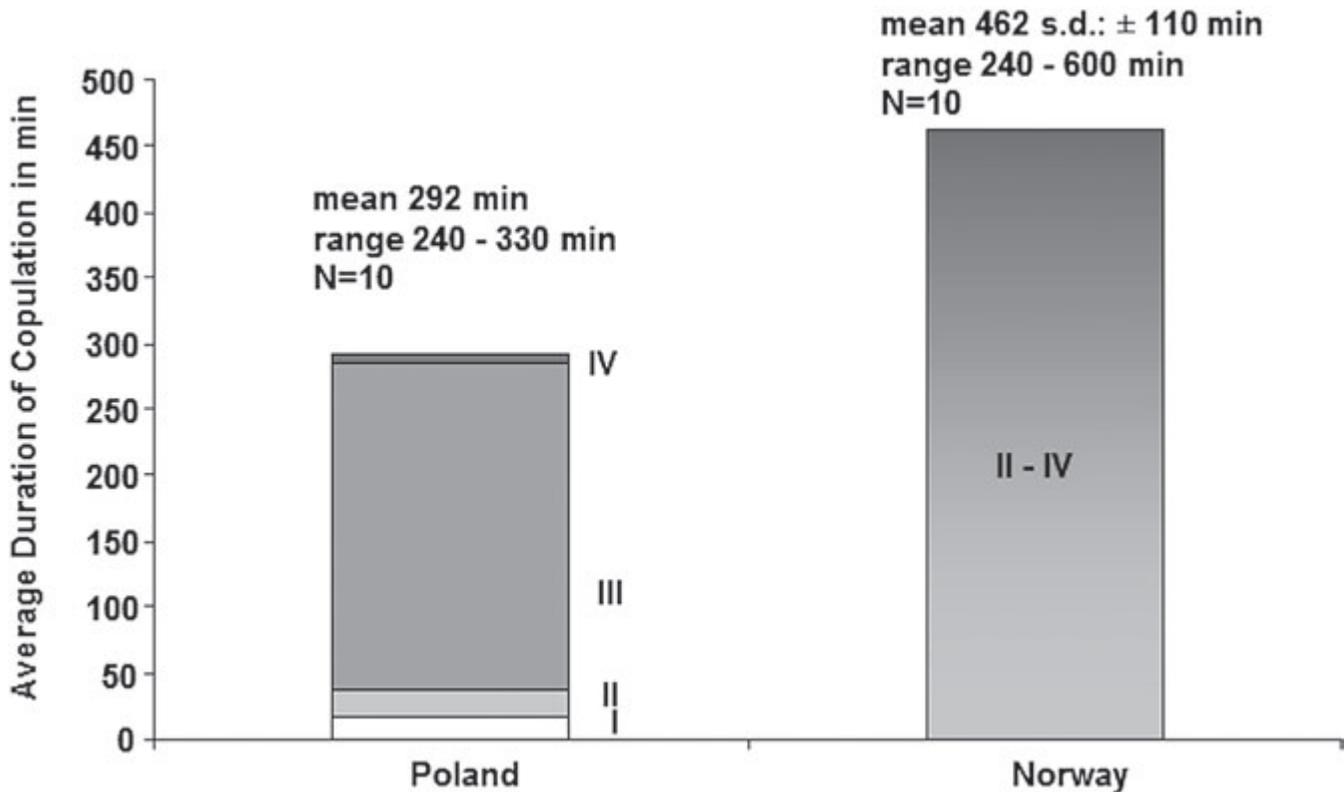
*Abstract* *Arion vulgaris* Moquin-Tandon 1855 (synonym *Arion lusitanicus* Mabille 1868) has been spreading through Norway during the last 20 years. We present phenological and reproductive data from western Norwegian populations. Mating experiments under semi-natural conditions revealed a prolonged duration of copulation compared with other studies. In partner choice experiments using marked slugs we observed that remating occurred in this *Arion* species. For the first time, experimental evidence for mating between *Arion rufus* x *ater* (Linnaeus 1758) and *A. vulgaris* is reported which confirms other genetic and morphological studies suggesting low reproductive isolation within the larger *Arion* species complex. Moreover, even based on very preliminary experiments our data suggest a case for possible mitochondrial heteroplasmy in *Arion*.

*Key words* slug, mating, *Arion* species complex

## INTRODUCTION

The problems involving invasive species are increasing throughout the Northern Hemisphere mainly due to anthropogenic introductions. The invasive slug, *Arion vulgaris* (also regarded as *Arion lusitanicus* Mabille 1968, see below), originates from SW Europe, and has been introduced to large parts of Europe including Scandinavia (Proschwitz, 1992). *Arion vulgaris* was first reported from Norway in 1988 (Proschwitz & Winge, 1994) and has since become a major pest, especially along the coast of western Norway (Dolmen & Winge, 1997; Hofsvang, 2003). This invasive slug currently occurs in large populations as far north as Nord-Trøndelag and is spreading in eastern and northern Norway (Dolmen & Winge, 1997, unpubl. data). One general characteristic of this species is its high variability in life history and, in particular, in reproductive biology (see e.g. Kozłowski, 2007). Here we present some preliminary data on reproductive parameters such as lifespan, mating season, copulation time, re-mating and clutch size from *A. vulgaris* populations in Norway, and discuss our results in comparison to other field and experimental studies.

Self-fertilization, hybridisation and inconsistency in morphological and molecular characters are important topics in the ongoing debate about the taxonomy and systematics in the large-sized *Arion* species complex (Foltz *et al.*, 1982; Noble, 1992; Noble & Jones, 1996; Campbell, 2000; Quinteiro *et al.*, 2005; Geenen, Jordaens & Backeljau, 2006). For example, the status of *A. ater* and *A. rufus* as subspecies (Burnet, 1972; Evans, 1986) or as species in their own right (Anderson, 2005; Bank, Falkner & Proschwitz, 2007) is still under debate. Moreover, there is confusion over the taxonomic status of *Arion lusitanicus* Mabille 1868 and *Arion vulgaris* Moquin-Tandon 1855 in the malacological literature. Some workers suggest that Mabille's taxon should be restricted to an *Arion* (*Mesarion*) species that is endemic to the Iberian Peninsula and that the widespread synanthropic pest should be called *A. vulgaris* (Anderson, 2005; Quinteiro *et al.*, 2005; Proschwitz, 2009). Recent studies (e.g. Proschwitz, 2009, Hatteland *et al.*, submitted) confirmed this opinion and so we use *Arion vulgaris* here but we are aware that the taxonomic status of the slug species remains unresolved although suggestions have been made on how the situation can be resolved (Kadolsky, 2012).



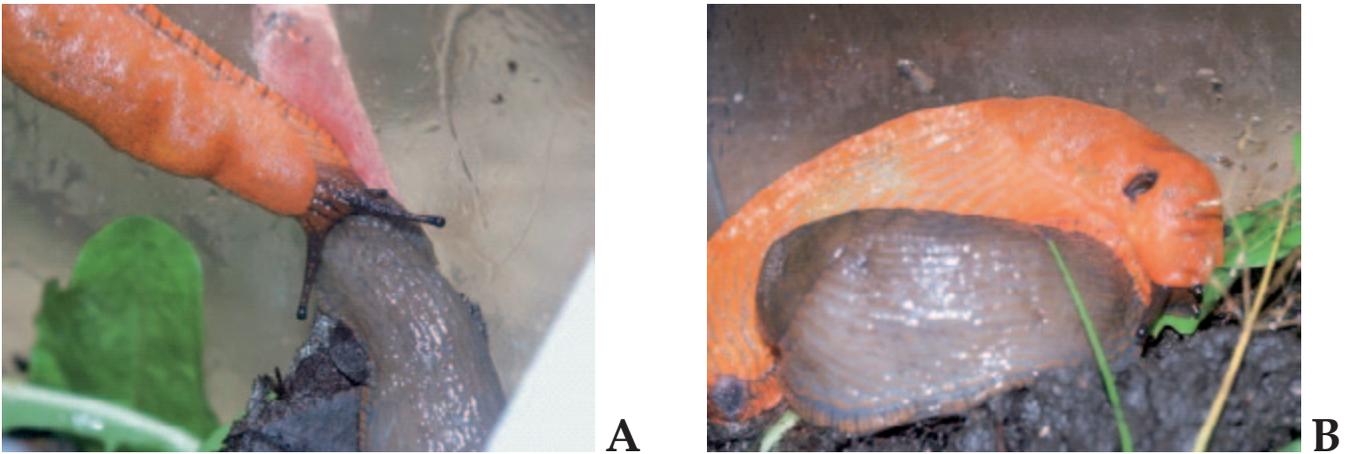
**Figure 1** Duration of copulation time in *Arion vulgaris* from 2 different mating experiments from Poland (Kozłowski & Sionek, 2001) and Norway (this study). I-IV refers to different mating phases – see text for more details.

Evans (1986) assumed hybridisation and introgression between *A. ater* and *A. rufus*. There is evidence that *A. ater* and *A. rufus* belong to a species aggregate with a high degree of hybridisation in Scandinavia (Noble, 1992; Hatteland *et al.*, submitted). Hagnell, Schander & Proschwitz (2003) clearly distinguish between *Arion ater*, as a native species, and *A. rufus* as an introduced species. In western Norway, *A. rufus* was recorded in one site in the vicinity of Bergen during 1850–1900 (Friele, 1853; Økland, 1922) and may have crossed with *A. ater* (Hatteland *et al.* submitted). However, the most likely explanation is that hybrids of *A. ater* and *A. rufus* have been introduced to Norway recently, which may also explain why hybrids seem to be so common in western Norway (Hatteland *et al.*, submitted).

Hagnell *et al.* (2003) propose hybridisation between *A. vulgaris* and *A. ater* in Sweden and recent molecular studies also suggest introgression between these two taxa (Hatteland *et al.*, submitted). Here, for the first time, we present experimental evidence for mating between *Arion vulgaris* and *A. rufus* × *ater* hybrids.

## METHODS

*Species Determination* There is pronounced colour variation in Scandinavian specimens of *Arion vulgaris* but the chocolate-brown morph is the most common one in western Norway (Hatteland *et al.*, submitted). To avoid confusion with *A. ater*, only such coloured specimens were used for the mating experiments and all dark brown and blackish specimens were excluded. However, colour variation changes over season and older individuals getting pale or grey and their fringe may change into a more orange colour as well. However, brownish and grey forms of *A. vulgaris* have in most cases a pale-brownish or brown fringe. In contrast to Noble's (1992) observation, specimens of *A. vulgaris* with orange foot fringe occasionally occur in Norway (see figure 2), and, their differentiation from *A. rufus* (and its hybrid forms) can be difficult. Therefore, we cannot exclude that some of our studied individuals were to some degree hybrids of the *Arion vulgaris-ater-rufus* complex. We did not perform morphological or genetic analysis with specimens



**Figure 2** Mating between *Arion rufus x ater* hybrid (orange colour) and *A. vulgaris* (Experiment I – see Table 1): A) Initial phase where slugs follow each other and B) copulation with circling.

assessed as *A. vulgaris* with the exception of the mating experiment involving different lineages. We attempted to cross *A. vulgaris* with hybrids *A. rufus x ater*. *Arion vulgaris* was collected in western Norway, while *A. rufus x ater* hybrids were collected from Denmark (hereafter called *rufus x ater* I and II) and near Trondheim, Norway (hereafter *rufus x ater* III) but the latter were probably recently introduced from Danish populations (Silkeborg, Jutland) as well (Proschwitz & Andersen, 2010). Before the experiment we assigned species to operational taxonomic units by characterising orange forms with an orange foot fringe as *A. rufus x ater*, and, chocolate-brown morphs as *A. vulgaris* (see Noble, 1992). After the experiment was terminated the slugs were stored in 70% ethanol. Taxonomic status was verified by using morphological (atrium, free oviduct, ligula) and mitochondrial DNA (COI) as well as nuclear DNA (ITS) characters for individuals used in the mating between *A. rufus x ater* and *A. vulgaris*. COI sequences were obtained using the universal invertebrate primers LCO1490 and HCO2198 designed by Folmer *et al.* (1994), while ITS were amplified using a diagnostic multiplex PCR including species-specific primers designed by Hatteland *et al.* submitted. Morphological and genetic characters confirmed the correct determination of *A. vulgaris* (unfortunately genetic analysis was not possible for all individuals). Results were more complex for the putative *rufus x ater* hybrids. Shape, ornamentation and a second wall of the ligula suggested introgression of *A. ater* into *A. rufus* (L. R. Noble, pers. comm.), whereas the mitochondrial COI and nuclear ITS

indicated *A. rufus*. This finding is in concordance with a study on Danish populations of the *Arion ater*-aggregate which revealed a high degree of hybridisation (Noble & Jones, 1996). And more important here, individuals in this study showed mtDNA characteristic of one taxon but at the same time an intermediate genital anatomy. In addition, we tested eggs (i.e. 3–4 eggs per tested sampling) from four individuals of the crossing experiment (two *A. vulgaris*, two *A. rufus x ater*) with respect to mtDNA and ncDNA. In addition, eggs were screened using two multiplex PCRs with species-specific primers for *A. ater*, *A. vulgaris* and *A. rufus* amplifying COI and ITS fragments, respectively (Hatteland *et al.*, 2011, Hatteland *et al.* submitted).

*Experimental set-up* Mating experiments with *Arion vulgaris* were carried out under semi-natural conditions in an ordinary garden in August–October 2008. Several individuals (ranging from 2–8) were kept together for mating choice and remating. Each individual was collected from the garden itself or its close surroundings at the end of July. Since the experiments were designed as a methodological screening test for further studies, three different enclosures were used:

- Small cages (30×20×15 cm) for experiments with 2–4 individuals.
- Glass containers (40×20×20 cm) for partner choice experiments with eight individuals.
- A fenced area (50×50 cm) of grassland for partner choice experiments with six individuals.

For experiments with more than four individuals we used individual transponder markers according to the method described by Grimm (1996). In the small cage experiments with four individuals, the two remaining slugs were always removed after one pair had started mating to avoid disturbance. In mating choice experiments the number of slugs was constant ( $N = 6$  and  $N = 8$ , respectively) but in one case a slug showed indications of sickness and was replaced. In addition, crossing experiments (using small cages) with three separate pairs of *A. vulgaris* and *A. rufus x ater* were carried out.

The mating experiments were conducted between 20<sup>th</sup> August and 30<sup>th</sup> September 2008. Since mating in *Arion vulgaris* mostly occurs in late afternoon and at night time, from about 4 p.m. until 9 p.m. all cages were controlled for mating slugs in time intervals of 3–4 hours or less. All cages and containers were filled with soil. Soil was renewed and cages were cleaned at regular intervals of 3–4 days. Fresh food such as carrots, lettuce and cat food pellets was provided *ad libitum* every 2–3 days. Cages and glass containers were placed in shady areas of the garden and moisture was controlled and regulated by spraying water on demand daily.

## RESULTS AND DISCUSSION

*Life span, phenology and mating season* Recent studies in Norway have shown that *Arion vulgaris* hatch in autumn and overwinter as juveniles and sub-adults showing a high variability in both size and biomass (Dirks *et al.*, in prep.). Overwintering of adult *A. vulgaris* happens rarely in western Norway (unpubl. data). The species has also been recorded overwintering as eggs in Poland (Kozłowski, 2007) and in Denmark (Pagh & Jensen, 2008), but not in Norway (unpubl. data).

In field surveys, the number of recorded individuals peaks in May and decreases during the summer (Dirks *et al.*, in prep.). The weight of overwintering juveniles and sub-adults varies markedly ranging from <1 g up to 12 g but most slugs (>80%) are less than one gram (Dirks *et al.*, in prep.).

Mating in our experiments occurred from the end of August until the end of September, which is in accordance with previous studies in western Norway (unpubl. data). The earliest observed

mating in Norway, however, is from a rather northern locality (Trælsnes, Sømna District, Nordland – County; Decimal Degrees: 65.190° North/12.103° East) and was on the 13<sup>th</sup> July 2008 (T. Waerstad, *in litt.*). Most individuals are alive until the end of October/middle of November but from the end of September some specimens change to a greyish colour or develop a pale complexion and die soon afterwards. Egg laying is observed from the end of August until the end of October in captivity as well as in fields.

*Egg number and clutch size* Low predation mortality due to sticky mucus (but see Hatteland *et al.* 2011), high fecundity, catholic feeding and rapid growth rate of *A. vulgaris* are considered three of the key factors for its rapid spread in Europe and high impact as a severe pest species (e.g. Kozłowski, 2007), although studies on individual reproductive outcomes are surprisingly rare. Published values vary from 50–550 eggs/individual (Quick, 1960; Grimm, 1996; Briner & Frank, 1998; Kozłowski, 2000). A detailed study from Poland revealed 243–541 eggs per individual under field conditions, and 122–382 eggs per individual under laboratory conditions (Kozłowski, 2000).

In our mating experiments with marked slugs, the maximum numbers of eggs per individual was 157 (mean 101, s.d. 33,  $N = 9$ ). This relatively low number might indicate sub-optimal conditions as reported from other studies with reared Iberian slugs in captivity (see Davies, 1987; Kozłowski, 2000). Nevertheless, our results are comparable to Grimm's (1996) study using marked *A. vulgaris*, in which 80–90 eggs/individual were found.

Hatteland & Baldeweg (unpubl. data) studied density of eggs from 8 different sites in western Norway and found high variability in the number of eggs per clutch, ranging from less than 5 to 144 (mean 51 eggs/clutch; number of clutches, 155), which is in accordance with Kozłowski's (2000) data of 60–70 eggs/clutch. One has to keep in mind that *A. vulgaris* usually lay more than one clutch (typically 3–5) (Kozłowski, 2000), therefore a maximum number of 150–250 eggs/individual can be estimated.

Our results and data from Proschwitz (1992, 1994), who found up to 400 eggs/individual, suggest that the reproductive output of *A. vulgaris* in the most northern parts of their distribu-

tion seems to be similar to that in other regions in western and central Europe.

### Mating – experiments

1. *Duration of copulation* Mating behaviour of *A. vulgaris* can be divided into four phases according to Kozłowski & Sionek (2001):

- I initial phase with partner finding and recognition (10–24 minutes),
- II courtship dance with circling, eversion of and connection of atria (about 20 minutes),
- III copulation with spermatophore transfer and starting the retraction of copulatory organs (3–5 hours), and,
- IV post-copulation with full retraction of the atrium and separation (less than five minutes).

In one of the few studies on mating behaviour in *A. vulgaris*, Davies (1987) reported that the mating act lasts about 140–150 minutes, but based on fragmentary observation. In a substantial experimental investigation, Kozłowski & Sionek (2001) found that complete copulation, i.e. from finding a partner until separation, lasts about 4–5.5 hours (mean 292 min, N = 10). In our study, however, we found a much longer duration of copulation of 7.7 hours on average, ranging from 4–10 hours (N = 10) (Fig. 1). It has to be noted that the duration of the first phase (partner finding and recognition) is not included in our figures because the beginning of the initial phase was not often observed exactly. Other, more fragmentary observations ranged from 1–8.5 h (mostly 4.5–5.5 h, N = 20), and these are minimum values because the precise time of courtship dance or separation was not observed.

There are three main differences between our experimental set-up and that of Kozłowski & Sionek (2001) which may contribute to the observed prolonged duration of copulation: 1) ambient temperature, 2) number of individuals per cage, and, 3) the use of marked individuals in our study. Unfortunately, Kozłowski & Sionek (2001) presented no copulation time for the single mating events which prevented a statistical comparison between our and their data.

Kozłowski & Sionek (2001) gave no exact date of their mating experiments which probably started in the second half of July (Kozłowski, 2000), but they mention rainy weather and night-time air temperature of 14–17°C. Kozłowski (2000) states temperatures below 10°C will limit, and below

5°C preclude, copulation. Kozłowski & Sionek (2001) reported prolonged mating of 1–2 hours in shady places and that full sun exposure interrupted mating. In contrast, our experiments were carried out under a lower ambient temperature (5.5–16.1°C: Norwegian Meteorological Office: station Bergen-Flesland), but without direct sun exposure to cages and containers. The daily minimum temperatures during our experiments had fairly low extreme values (16 August 2008, 6.2°C; 24. September 2008, 5.5°C) that could limit copulation but definitely did not preclude copulation. For example, mating was observed at temperatures of 4°C and below in the laboratory (personal observations and L. R. Noble, pers. comm.).

The average duration of copulation increased with the density of slugs: 2–4 individuals/cage, mean 405, s.d. 105.2 min., N = 6; versus 6–8 individuals/cage, mean 547.5 s.d. 37.7 min., N = 4. In addition, individuals kept at higher densities were marked with transponders and effects of the markings on reproductive behaviour cannot be excluded (but see Grimm, 1996). However, the low numbers of replicates for the two density treatments do not allow a stronger conclusion to be reached from our preliminary data.

2. *Partner choice* In two partner choice experiments, a constant number of 6 and 8 individuals were kept together, respectively. Fourteen out of 15 slugs (one was replaced but did mate before – see methods) did mate. Of these, 6 individuals mated once, 6 twice and two mated three times. Among the 12 copulations that were observed, 6 concerned remating individuals. Of these, three mated to an unmated partner and three to a previously mated partner. In the latter three cases both partners remated with a new partner. During successive matings, the number of unmated slugs became smaller, so the opportunity for choosing an unmated partner became more limited.

Preliminary studies (unpubl. data) suggest that size differences between partners (e.g. <4 cm versus >8 cm length) did not affect choice and mating behaviour (see also Kozłowski & Sionek 2001). The seasonal development of reproductive organs of *A. vulgaris* has been studied (unpubl. data) using the methods described in Smith (1966) and showed that in late summer the female tract has reached sexual maturity in all adult individuals independent of size and weight. Nevertheless,

**Table 1** Verification of species identity of individuals of crossing mating experiments based on genital morphology (see methods), and, genetic analyse of mitochondrial COI and nuclear ITS for both slugs and their eggs. Genbank numbers of submitted sequences are given in brackets.

Character	Experiment I		Experiment II		Experiment III	
	Partner Ia	Partner Ib	Partner IIa	Partner IIb	Partner IIIa	Partner IIIb
Genital Morphology	<i>vulgaris</i>	<i>ater x rufus</i>	<i>vulgaris</i>	<i>ater x rufus</i>	<i>vulgaris</i>	<i>ater x rufus</i>
mt COI adults	<i>vulgaris</i>	<i>rufus</i>	not studied	<i>rufus</i>	not studied	<i>rufus</i>
nc ITS adults	<i>vulgaris</i>	<i>rufus</i>	not studied	<i>ater, rufus</i>	not studied	<i>rufus</i>
mt COI eggs	<i>rufus</i> (JF 950511)	<i>rufus</i> (JF 950512)	no eggs laid	PCR failed	<i>vulgaris</i>	<i>rufus</i>
nc ITS eggs	<i>rufus</i>	<i>rufus x vulgaris</i>	no eggs laid	PCR failed	no band	<i>rufus</i>

it has been observed that slugs do not have to be fully grown for mating activity (Wiktor, 1989 cited in Kozłowski & Sionek 2001).

**Crossing experiments** In cage experiments, three separate pairs (I-III) of *A. vulgaris* and *A. rufus x ater* were kept together from the 13<sup>th</sup> August until the 30<sup>th</sup> October. Mating was observed on the 13<sup>th</sup> (pairs I and II) and the 20<sup>th</sup> (pair III) August and took about four hours (see fig. 2). None of the *A. rufus x ater* hybrids re-mated; neither with the same partner nor with other individuals of *A. vulgaris* (a new potential partner was offered in September for 4–5 days).

Two individuals of *A. vulgaris* and all three *A. rufus x ater* laid eggs (*A. vulgaris* I:157 / III:126, *A. rufus x ater* I:136 / II:64 / III:63). For unknown reasons none of these eggs hatched, but both Davies (1987) and Kozłowski & Sionek (2000) point out that fecundity and hatching of slugs may vary depending on various factors. Species identity based on morphological and genetic analysis of individuals is shown in Table 1. Sequences of mitochondrial COI were obtained with a length of about 630 base pairs and were compared both with Genbank sequences and unpublished data of the large *Arion* taxa complex (Hatteland *et al.*, submitted).

As far as we are aware, our results provide the first experimental evidence for potential hybridisation between *A. vulgaris* and hybrids of *A. rufus x ater*. Our observations confirm the high tendency of hybridisation and low reproductive isolation within the larger *Arion* species complex. This is in concordance with observations about the lack of a mating barrier between *A. vulgaris* and *A. ater* (Hagnell *et al.*, 2003; Hatteland *et al.*, submitted) and *A. ater* and *A. rufus* (Noble, 1992; Noble & Jones, 1996) in Scandinavia.

Considering the wide extent of hybrids of *A. ater* and *A. rufus* in Sweden and Denmark (Noble & Jones, 1996), our results indicate a mixed breeding system within the three large *Arion* species in Scandinavia. Direct observation of crossing events in the field with subsequent identification of mating partners seems to be missing. But there is indirect evidence for hybridisation based on change of genital structures and external appearance (colour type) both for *A. vulgaris x A. ater* in Scandinavia (Hagnell, Schander & Proschwitz (2004), Proschwitz & Andersen 2010), and, *A. vulgaris x rufus* in Germany (Proschwitz, *in litt.*).

We tested gene sequences of parental slugs and its eggs from our crossing experiments (see Table 1). For three individuals (two *A. rufus x ater*, one *A. vulgaris*) the distinctive sequences of the analysed mitochondrial COI gene fragment of the parents were also found in their eggs. Distinctive means here a complete concordance of the 720 base pair (bp) long fragment of the mt COI gene as found for *A. vulgaris* and *A. rufus* in other studies in Scandinavia (Hatteland *et al.*, 2011, Hatteland *et al.*, submitted).

One noteworthy finding, however, is a discrepancy between one of the two tested gene sequences of an individual of *A. vulgaris* and its offspring (Partner Ia, Table 1). Whereas, the COI gene sequence of the parent was distinctive for *A. vulgaris*, the subsampling of its eggs had a distinctive *A. rufus* sequence (see table 1). This result was confirmed for another subset of eggs. Remarkably however, different results were obtained for the three subsets of eggs when using a diagnostic multiplex PCR with species-specific primers for *A. vulgaris*, *A. ater* and *A. rufus* amplifying fragments of the COI gene (see Hatteland *et al.* 2011). Two of the three subsets of eggs showed a species-specific *A. vulgaris*

band (310 bp), whereas for the third subset an *A. rufus* specific band (362 bp) was found (data not shown). We suspect, that our result might indicate a case of mitochondrial heteroplasmy. That is the coexistence of *A. vulgaris* and *A. rufus* mitochondrial haplotypes in a single organism. Heteroplasmy has been regarded as a rare phenomenon in animals, but it is probably more widespread than previously assumed exemplified by recent studies of mussels, insects, birds and mammals (Ballard & Whitlock 2004; Van Leeuwen *et al.*, 2008; White *et al.*, 2008; Magnacca & Brown, 2010). Many cases come from interspecies hybridizations, and breakdowns of mechanisms to recognize and remove paternal mtDNA have been discussed (White *et al.*, 2008 and references therein). It has to be kept in mind, that we analyzed egg clutches and not single eggs. But detection of heteroplasmy strongly depends on the developmental state of the eggs (Fontaine *et al.*, 2007), and consequently on the ratio of maternal to parental mtDNA. Since, we had no control of the development of the eggs and used egg clutches this might explain different results in the multiplex PCR.

However, the PCR-based methods used here may be susceptible to NUMTs (i.e. non-functional copies of mitochondrial (pseudo-) genes in the nuclear chromosomes) whose transmission is biparental. For this, each species would need to have an exclusive NUMT not found in the other species. More important, this exclusive NUMT would need to match the paternal mitochondrial sequence of the cross exactly i.e. an *A. rufus* pseudogene in *A. vulgaris* individual (see Fontaine, Cooley & Simon, 2007 for a similar problem). We consider this possibility to be highly unlikely to explain our results or those found in other comprehensive studies with *Arion* taxa (see Hatteland *et al.*, 2011, Hatteland *et al.*, submitted).

In summary, we interpret the findings of different mt DNA 1) between a maternal *A. vulgaris* and its eggs, and, 2) among egg clutches from the same individual, as strong indication for mt heteroplasmy. However, we are aware of the limited experimental basis for our conclusion. Therefore, we do not argue here that mt heteroplasmy is proven in *Arion* rather that it is a subject for further experiments.

The picture is slightly different for the nuclear ITS-gene, and is in concordance with similar

studies in Scandinavia showing introgression between different *Arion* taxa (Hatteland *et al.*, subm.). We found dominance of *rufus* genes in eggs in all cases (regardless of the ITS of the parents) with the exception of a single *rufus x vulgaris* hybrid sequence, which remarkably derived from a *rufus x ater* parent (experiment I).

However, in order to disentangle hybridisation and the speciation processes in the large *Arion* complex an approach is needed that combines morphological, genetic and experimental methods using a wide geographic sampling. Geenen *et al.* (2006) studied similar processes for the closely related *Carinarion* complex and discussed the resulting problems and challenges for nomenclature, taxonomy, and species concepts.

Interestingly, and for unknown reasons, *A. ater* seems to disappear in areas where *A. vulgaris* has invaded in Norway (unpubl. data). A similar process has been reported for *A. rufus* replacing (probably without any hybridisation events) *A. ater* from the Island of Öland (Proschwitz & Andersen, 2010). Population genetic studies and further crossing experiments may shed light on these matters and could help resolve this species complex as well as tracing the impact of the invasive Iberian slug.

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