A method of culturing and breeding slugs through several generations

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There is very little published information about how to culture slugs. There are at least 36 species of slug in the UK (Anderson, 2008) and as many as 44 (Rowson *et al*., 2014), many of which are nonnative (Cameron, 2016). They are important in terms of causing economic damage to crops (South, 1992), as well as hosts for medically important parasites such as *Angiostrongylus cantonensis* (Barratt *et al*., 2016). The aim of our study was to discover whether three species of slug, *Deroceras invadens* Reise, Hutchinson, Schunack & Schlitt 2011, *Arion hortensis* Férussac 1819 and *Ambigolimax valentianus* (Férussac 1822), could be cultured and mated to produce offspring and whether these offspring would be viable enough to produce more progeny. Ultimately, by providing information on how to maintain and breed slugs through several generations, perhaps a 'model slug' could be developed, which could benefit an array of subjects including genetics, genomics and developmental biology.

Preliminary observations in the laboratory showed that *D. invadens* and *A. hortensis* preferred to lay eggs in peat-free compost (SylvaGrow®, Melcourt, UK) as compared to garden bed or turf soil. Observations also demonstrated differences in egg development for the three species at different temperatures. Specifically, *D. invadens* at 10 °C, and *A. hortensis* and *A. valentianus* at 15 °C produced the highest level of viable eggs, presumably due to differences in the ecological niches they inhabit. The slugs were collected from a garden in Maghull, Liverpool (UK OS grid reference SD373027). They were kept in nonairtight plastic boxes (35 x 23 x 22 cm) at 5 °C with moistened paper and fed lettuce *ad libitum* for 1 week before use to check for any signs of ill health. Compost (10–15% moisture content) was added to a

height of 3.5 cm to a total of 60 50-ml universal bottles. Two adult *D. invadens*, *A. hortensis* (all animals collected were >0.10 g in weight and >2.5cm in total length) or *A. valentianus* (>0.2g and >3.5cm) were added to each tube, with 20 universal bottles, each with two slugs, being used for each slug species. Tap water (0.5 ml) was added to each tube, a ball of cotton wool was then added and the lid was loosely closed. The tubes with *D. invadens* were stored in an incubator at 10 °C and those with *A. hortensis* and *A. valentianus* were incubated at 15 °C. After 8 d, every clutch of eggs in each tube was weighed and the slug eggs were counted; these were then transferred to 10-cm Petri dishes (*c.* 60 eggs per dish) with pre-moistened filter paper with a small amount of compost substrate and sealed with Parafilm®. The dishes were incubated for up to 20 d at 15 °C. Egg hatching occurred generally between 14 and 20 d. After hatching, *c.* 60 neonate slugs were transferred to container (30 x 10 x 10 cm) of compost (previously frozen at -80 °C overnight to kill any metazoan parasites). The container was kept at 50% humidity (monitored using a Tinytag, Gemini Data Loggers, UK) and the soil kept between 10 and 20% water content (monitored using a soil moisture tester, Xiaomi, China). The container was misted with distilled water once a week. Slugs were fed a mixture of iceberg lettuce, carrots and calcium tablets every 2 weeks. Any remaining rotten food was removed every few days. Once the slugs had reached *c.* 5 mm they were transferred to containers containing 30 slugs of the same species and kept under the same conditions. After 4 months, most adult *D. invadens* and *A. hortensis* had reached a weight of >0.10 g and most adult *A. valentianus* were >0.20 g, and so were treated as having reached sexual maturity. They were mated using the same protocol previously mentioned. This process was repeated for the second and third generation of all three species.

A one-way ANOVA was used to analyse whether there were significant differences between the numbers of eggs and the weight of eggs laid in generations 1, 2 and 3 for each of the three slug species and between the numbers of eggs laid by the three different slug species in generations 1, 2 and 3.

There was no significant difference between the numbers of eggs or the weight of eggs produced by generations 1, 2 and 3 of *D. invadens*, *A. hortensis* and *A. valentianus* ($P > 0.05$; Table 1). All three slug species produced similar numbers of eggs and there were no significant difference in the numbers of eggs produced by *D. invadens, A. hortensis* and *A. valentianus* in generation 1 ($P = 0.80$), 2 ($P = 0.70$) or 3 ($P = 0.447$) (Table 1).

Using three different species from three genera, we have here demonstrated that slugs can be routinely mated under laboratory conditions to produce similar clutches of eggs over several generations. An important question to consider is whether continuous culturing may affect the health of the slugs due to several generations of potential inbreeding. However, we observed no reduction in egg number or health of the slugs over the three generations. Specifically, the numbers of eggs did not vary over the three generations, with *D. invadens* producing between 25 to 28 eggs, *A. hortensis* between 21 to 24 and *A. valentianus* between 18 to 20. Although it has been reported that the number of eggs may vary with species (South, 1992), we did not observe any differences between the three species used. The numbers of eggs laid in our study by each species is similar to other studies. For example, Carrick (1938) found *D. reticulatum* could lay up to 500 eggs a year with a mean of 22 eggs per batch (range: 9–49 eggs). However, that estimation is based on field studies. In contrast, in captivity, Davies (1977) found the number of eggs laid by *A. hortensis* ranged from 10 to 30 per batch.

Other studies that have attempted to rear slugs focused on using an array of different substrates. For example, Sivik (1954) used wooden trays with gauze and soil; Stephenson (1962) used a combination of loam soil, peat and sand in screw capped jars; and Kingston (1966) used fine gravel or moistened filter paper (with blackboard chalk as a source of calcium). Vermiculite has also been used (Gray *et al*., 1985) as a substrate. Our slugs were housed in nonairtight plastic boxes with ample moisture and a thin layer of compost. When used for mating they were placed in 50-ml universal bottles with compost, which consistently yielded similar numbers of eggs with no reduction in viability.

Maintaining consistent and correct moisture content is an important factor in slug rearing. Arias & Crowell (1963) found that *D. reticulatum* produced a maximum number of eggs in soils at 75% saturation and no eggs in soils at 10% saturation. However, Willis *et al*. (2008) showed that *D. reticulatum* the greatest number of eggs were laid in soils at 53% saturation. The compost used in our study initially had moisture content of 10–15%; a further 0.5 ml of water was applied later through spraying directly into tubes, increasing the compost moisture saturation to between 15 and 20%.

Another important factor to take into account when rearing slugs is diet. There are many diets that have been used to rear slugs including breakfast cereal, leaf litter

and fungi (Cook & Radford, 1988), oat bran (Howlett *et al*., 2009), dog food and fresh fruit (Hamilton *et al*., 2020). Synthetic diets of calcium alginate beads have also been used to rear slugs, but reproductive output was poor (Wright, 1973). As *D. invadens, A. hortensis* and *A. valentianus* are all generalist herbivores (South, 1992), we fed them a mixture of lettuce, carrot and calcium tablets. Our results demonstrate that on this diet, the slugs were able to grow and mature quickly with no reduction in reproductive output. We gave the slugs a choice of foods as previous research has shown they choose the food type that contains the nutrients they lack the most (Cook *et al*., 1999). Carrot is a particularly good choice of food for laboratory-based studies as compared to potato, lettuce, apple and bran, which decay quickly causing microbial contamination that may affect the health of the slugs (Stephenson, 1962).

A continuous culture of slugs could aid in research on slug genomics (Chen *et al*., 2020), transcriptomics (Ahn *et al*., 2017), behaviour (Kozlowski *et al*., 2016), microbiome analysis (Reich *et al*., 2018), feeding (Barone & Frank, 2008) or novel molluscicide screening (Klein *et al*., 2020). Also, 'sterile' slugs (i.e. those free of metazoan parasites) could be used in coevolutionary studies with common parasites such as trematodes, parasitic flies or mites (South, 1992). For example, we are particularly interested in using these slugs for infection and coevolutionary studies using the slug parasitic nematode *Phasmarhabditis hermaphrodita* (Cutler & Rae, 2020). In summary, the successful culturing and mating of slugs may allow these animals to be developed as model gastropod study organisms.

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Table 1. Reproductive output of slugs *Deroceras invadens*, *Arion hortensis* and *Ambigolimax valentianus* over three generations, parental (P), F1 and F2.

Generation	**No. of pairs**	**No. of clutches**	**Mean no. of eggs per pair**	**SE**	**Range**	**Mean clutch weight (g)**	**SE**	**Mean egg weight (g)**
D. invadens								
P	20	19	26.5	2.7	5-50	0.10	0.01	0.004
F1	20	18	28.6	2.8	10-54	0.16	0.05	0.006
F2	20	18	25.4	2.4	11-50	0.09	0.01	0.004
A. hortensis								
P	20	17	21.1	2.4	4-36	0.14	0.02	0.007
F1	20	17	24.3	2.9	3-56	0.14	0.02	0.006
F2	20	16	21.7	2.7	3-40	0.14	0.02	0.006
A. valentianus								
P	20	18	18.4	2.1	2-33	0.19	0.02	0.01
F1	20	18	19.7	2.2	5-38	0.21	0.02	0.01
F2	20	18	20.9	2.9	2-55	0.19	0.02	0.01