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1 **Title Document**

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3 Terrestrial slugs (Mollusca: Gastropoda) share common anti-predator defence mechanisms but their
4 expression differs between species

5

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1 Terrestrial slugs (Mollusca: Gastropoda) share common anti-predator defence mechanisms
2 but their expression differs between species

3 **Abstract**

4 Terrestrial slugs (Mollusca: Gastropoda) are an interesting group in which to study anti-
5 predator behaviour because few predators are mollusc-specialists. Ground beetles
6 (Coleoptera: Carabidae) are important slug predators and slugs appear to possess general
7 primary and secondary defence mechanisms when carabids are encountered. Slugs may avoid
8 areas recently visited by carabids (primary defence) or, when attacked, exude highly viscous
9 mucus (secondary defence). Slugs are, however, a polyphyletic group which are present in
10 diverse habitats with varying exposure to potential predators. Therefore not all slugs might
11 possess common anti-predator defences. This study examined whether avoidance of cues
12 from *Pterostichus niger*, a generalist carabid predator, is a primary defence mechanism
13 shared by a number of slug species representing different taxonomic families and varying
14 degrees of ecological specialization. Additionally we examined whether mucus chemistry is
15 altered in response to predation attempts, and whether this is a secondary defence common to
16 multiple species. We found that calcium, a vital element for molluscs, is secreted in greater
17 amounts when slugs are attacked. Choice experiments with *P. niger* demonstrated that
18 calcium had no effect on beetle feeding behaviour but that high-viscosity mucus deterred
19 feeding. We discuss how calcium secretion may contribute to mucus viscosity and represent
20 an effective anti-predator defence mechanism common to slugs. Results from no-choice
21 feeding trials suggest that carabid beetles can prevent slugs from exuding defensive mucus by
22 attacking the mantle region. Overall, we show that slugs share common primary and
23 secondary defence mechanisms but that the expression of these anti-predator defences varies
24 between species, reflecting the natural encounter rates of each slug species to predation in the
25 wild. Additionally, some species-specific anti-predator traits were observed which cannot be
26 satisfactorily explained as responses to carabids alone, which highlights the importance of
27 considering multiple predators when interpreting anti-predator behaviour in prey animals.

28

29 Keywords: anti-predator adaptation, predator avoidance, chemical defence, ecological
30 specialization, predation

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1 **Introduction**

2 Predation has long been recognised as an important selective force in the evolution and
3 maintenance of multiple traits in prey animals. Predation directly affects both resource use
4 and habitat choice in prey populations (Verdolin, 2006) and imposes selection for anti-
5 predator adaptations in the morphology and behaviour of prey species over evolutionary
6 timescales (Vermeij, 1994). Terrestrial slugs (Mollusca: Gastropoda) are an interesting group
7 in which to examine predator-prey relationships because, although very few animals appear
8 to specialize in malacophagy (mollusc-eating), terrestrial gastropods are nevertheless killed
9 and eaten by diverse species representative of many major terrestrial taxa (Barker, 2004).
10 There is much evidence to suggest that ground beetles (Coleoptera: Carabidae) are important
11 predators of terrestrial slugs (Symondson *et al.*, 2002a). Even generalist carabids, of which
12 slugs may form only a small part of the diversity of prey species consumed, have been shown
13 to influence slug densities under natural field conditions (Symondson *et al.*, 2002b). The
14 immediate fitness consequences of predation on prey animals (i.e. death) is so severe that
15 almost all animals must avoid predation, either via behavioural decision-making or
16 morphological defence mechanisms, to survive and increase their reproductive success
17 (Blumstein, 2006). Anti-predator defence mechanisms in prey animals are often broadly
18 defined as either primary or secondary defences (Edmunds, 1974). Primary defences can be
19 defined as behavioural responses or morphological adaptations which minimise the likelihood
20 of a prey animal encountering potential predators, whereas secondary defences can be
21 defined as behavioural or morphological adaptations which increase the probability of a prey
22 animal defending itself successfully and escaping after a predation attempt.
23 Behavioural avoidance of potential predators appears to be one of the most common primary
24 defence strategies adopted by prey species across a number of taxa (Kats and Dill, 1998) and
25 there is some evidence to suggest that slugs are capable of detecting and avoiding carabids in
26 this way. For example, the terrestrial slug *Deroceras reticulatum* Müller, a major agricultural
27 pest, exhibits an anti-predator response to ground beetles in general, and is capable of
28 detecting residual chemical cues from a number of polyphagous carabid species (Armsworth
29 *et al.*, 2005; Bursztyka *et al.*, 2013; Bursztyka *et al.*, 2016). Behavioural avoidance of areas
30 recently visited by carabids appears to be the primary defence adopted by *D. reticulatum*, and
31 is also an important contributing factor to decision-making with regards to foraging
32 behaviour (Bursztyka *et al.*, 2016) and habitat use (Bursztyka *et al.*, 2013) in slugs, which is
33 especially interesting since anti-predator behaviour may not be expected to evolve in
34 response to generalist predators (Armsworth *et al.*, 2005). Whether this primary defence

1 mechanism (avoidance) is common to other terrestrial molluscs or is specific to *D.*
2 *reticulatum* remains unknown. Recent work has shown that a variety slugs and snails
3 (including *D. reticulatum*) will avoid areas inhabited by the pathogenic nematode
4 *Phasmarhabditis hermaphrodita* (Schneider) (Wynne *et al.*, 2016). Interestingly, however,
5 *Deroceras panormitanum* ([Lessona](#) and [Pollonera](#)), *Arion subfuscus* (Draparnaud) and *Arion*
6 *hortensis* ([Férussac](#)) will become attracted to areas inhabited by *P. hermaphrodita* when they
7 are infected – an example of parasite manipulation of host behaviour (Morris *et al.*, 2018).
8 Detection of potential predators is intuitively an adaptive strategy insofar as it allows prey
9 animals to avoid areas with a high probability of encountering predation. However, for slugs,
10 escape-driven dispersal is slow relative to the speed of their predators, so slugs should
11 possess secondary defence mechanisms when attacked by predators.

12 A number of studies have demonstrated the importance of mucus in this regard; with the
13 assumption that highly viscous slug mucus might deter predation by fouling the mandibles
14 and limb-joints of attacking carabids (Pakarinen, 1994; Mair and Port, 2002; Foltan, 2004).
15 Since mucus production is an energetically expensive process for terrestrial slugs (Denny,
16 1980; Henderson and Tribskorn, 2002), it should not be expected that individuals can
17 continuously produce copious, highly viscous mucus without incurring fitness costs. If the
18 production of viscous mucus has evolved as an effective secondary defence strategy in slugs,
19 its production and chemical composition should respond to external cues such as mechanical
20 stimulation via predator attacks. Indeed, gastropod mucus has been reported to change
21 consistency based on the behavioural state of the animal, such that mucus is a clear, low
22 viscosity gel when the animal is at rest and an opaque, highly viscous glue-like substance
23 when stressed, at least in some species (Campion, 1961; Rollo and Wellington, 1979;
24 Pakarinen, 1992). This suggests that terrestrial gastropods exhibit an attack-induced change
25 in the chemistry of their mucus which may constitute an effective secondary defence against
26 predators.

27 In a recent study, Braun *et al.* (2013) provided evidence that calcium directly contributes to
28 the stiffness of slug mucus. The authors demonstrated that when calcium-containing cross-
29 links of *Arion subfuscus* Draparnaud mucus were disrupted, the mucus became less viscous
30 and lost its glue-like properties. Since calcium is an essential element and limiting factor for
31 molluscs (Fournié and Chétail, 1984), it should not be expected that gastropods continuously
32 release calcium into their mucus at a constant and elevated rate without incurring fitness
33 costs. Instead, the release of vital elements such as calcium should be controlled by external
34 stimuli such as predation.

1 The behavioural ecology of predation is usually considered in terms of optimal foraging
2 theory (Schoener, 1971). Predation time for typical “cruise foragers” (O’Brien *et al.*, 1990),
3 like predaceous ground beetles, is usually divided into two components: search time and
4 handling time. Whereas there has been much theoretical and empirical research on the
5 former, search time (for example: Charnov, 1976; Wallin, 1991), there has been less
6 emphasis on the latter, handling time. When small predatory invertebrates like carabid beetles
7 feed on relatively large prey like slugs, handling time can make up a significant proportion of
8 the total foraging effort; especially if the prey animals possess effective anti-predator defence
9 mechanisms. An understanding of the feeding strategies of generalist carabids is therefore
10 important in relation to natural pest control (an important ecosystem service) for pestiferous
11 slugs or when the prey are species of conservation concern, such as those examined in this
12 study.

13 This study aims to: (1) examine whether the detection and avoidance of residual chemical
14 cues from carabids is a primary defence mechanism common to multiple slug species; (2)
15 investigate, for the first time, whether predation attempts influence the defensive properties of
16 slug mucus by altering mucus chemistry, with particular interest in the role of calcium and
17 mucus viscosity in deterring predation by generalist carabids; and (3) examine the predatory
18 tactics of a generalist carabid in successfully killing and consuming slugs to determine
19 quantitatively the series of events leading to a successful kill (i.e. an analysis of the
20 “handling” phase of predation). In a number of behavioural assays we measured the primary
21 and secondary defence mechanisms of three terrestrial slug species representing distinct
22 taxonomic families and different degrees of ecological specialization. By examining the
23 multiple possible defence mechanisms from a number of habitat-specialist and generalist slug
24 species, it was possible to determine whether anti-predator responses might reflect the natural
25 degree of exposure to predation for each species, and the degree to which anti-predator
26 defence mechanisms may be common or species-specific.

27

28 **Materials and Methods**

29 *Description and collection of study organisms*

30 Primary and secondary defence mechanisms were studied in three terrestrial slug species,
31 each reflecting different taxonomic relationships, ecological niches and body sizes. The grey
32 field slug *Deroceras reticulatum* (Agriolimacidae; body length: 3-5cm) is an open habitat
33 specialist and a major pest of crops in the northern hemisphere where it is often the most
34 abundant slug in habitats where it occurs (Dedov *et al.*, 2006). The tree slug *Lehmanna*

1 *marginata* Müller (Limacidae; body length: 6-9cm) is an arboreal forest specialist which
2 tends to dominate the malacofauna in European woodlands (Jennings and Barkham, 1975;
3 Rowson *et al.*, 2014). The Kerry spotted slug *Geomalacus maculosus* Allman (Arionidae;
4 body length: 6-9cm) is a globally rare and internationally protected species occurring only in
5 the west of Ireland and north-western Iberia. It is a habitat-generalist which occurs in forests,
6 where it appears to be ecologically analogous to *L. marginata*, and in open habitats such as
7 blanket bog (Platts and Speight, 1988).

8 *Deroceras reticulatum* and *L. marginata* were collected using 0.25m² refuge traps
9 (DeSangosse, France) from different locations around Co. Galway, Ireland during May-
10 August 2015 for behavioural assays and predation trials; and during May-August 2016 for
11 collection of mucus samples for elemental analysis (described below). *Deroceras reticulatum*
12 was collected from traps placed on the ground on a patch of amenity grassland on the
13 National University of Ireland Galway (NUI Galway) campus (N53°16'45.9" W9°3'31.8" alt.
14 8m), and *L. marginata* was collected from traps placed on European beech *Fagus sylvatica* L.
15 trees at Barna Woods (N53°15'34.4" W9°8'0.1" alt. 32m). *Geomalacus maculosus* was
16 collected from traps placed on Sitka spruce *Picea sitchensis* Carrière in a conifer plantation
17 near Oughterard (N53°22'33.2" W9°24'40.9" alt. 173m) in December 2015 also using refuge
18 traps, and housed in captivity with permission from the National Parks and Wildlife Service,
19 Department of Arts, Heritage and the Gaeltacht (Licence No. C097/2015). All slugs were
20 housed in species monocultures in plastic containers (17cm × 11cm × 6cm) holding up to 5
21 individuals and were fed organic carrots for a minimum of one month prior to the collection
22 and elemental analysis of mucus (September – October 2016; discussed below).

23 The carabid beetle *Pterostichus niger* Schaller (body length: 1.6 – 2cm) is a generalist
24 predator with a Holarctic distribution (Hengeveld, 1980) which readily consumes live slugs
25 of all three species in captivity (*pers. obs.*). In Ireland, *P. niger* is highly abundant and often
26 dominates carabid assemblages in agroecosystems (O'Sullivan and Gormally, 2002), open
27 habitats such as mountain heath and blanket bog (McFerran *et al.*, 1995; Day, 1987), and
28 forested habitats (Williams and Gormally, 2010). Beetles were collected from a lightly-
29 wooded area on the NUI Galway campus between April and August 2016 (for no-choice
30 predation trials) and between April and August 2017 (for choice trials examining the
31 defensive mode of slug mucus) using dry pitfall traps covered with a corriboard sheet.
32 Beetles were then maintained in mixed-sex cultures at a density of five individuals per plastic
33 container (17cm × 11cm × 6cm) which contained a 2cm layer of moistened compost as
34 substrate, and a 3cm × 3cm section of corriboard as shelter. *Pterostichus niger* is a dark-

1 active species (Thiele, 1977). We therefore maintained beetle colonies on a 16:8hr light:dark
2 cycle so that predatory behaviour could be examined during the nocturnal phase of the
3 animal. Beetles were kept in these conditions and fed dried cat food pellets twice per week
4 for a minimum of one month before feeding trials were carried out (discussed below).

5

6 *Experiments*

7 (1) *Slug primary defence mechanisms*

8 *Deroceras reticulatum* exhibits behaviour consistent with an anti-predator kinesis in response
9 to residual cues from a number of generalist carabid beetles including *Pterostichus*
10 *melanarius* Illiger (Armsworth *et al.*, 2005), *Carabus nemoralis* Müller (Bursztyka *et al.*,
11 2016), *C. auratus* Linnaeus, *C. hispanus* Fabricius and *C. coriaceus* Linnaeus (Bursztyka *et*
12 *al.*, 2013). Clearly *D. reticulatum* can detect and avoid areas treated with residual scents from
13 diverse carabid species but the effect of such residual cues has not yet been tested on any
14 other slug species. We therefore measured the behavioural responses of *D. reticulatum*, *G.*
15 *maculosus* and *L. marginata* in the presence and absence of residual scents from *P. niger* to
16 determine whether avoidance of potential predatory beetles is common amongst different
17 slug species.

18 The methods used to examine the primary defence mechanism (avoidance) of each slug
19 species follow Armsworth *et al.* (2005). Trials were carried out in 21cm × 21cm wooden
20 arenas, the floor of which was covered in a base layer of dampened paper. Two additional test
21 papers, each measuring 10.5cm × 21cm were then prepared and placed over this base layer,
22 effectively dividing the experimental arenas into two treatment zones of equal size. Test
23 papers were prepared by allowing *P. niger* individuals ($n = 5$) to crawl over the moistened
24 paper inside a plastic container for 2 hours. Control papers were also moistened and placed
25 inside containers for 2 hours but were not exposed to any animals. Before being placed in
26 both beetle-exposed or control containers, all paper sections were sprayed 5 times from a
27 distance of approximately 30cm in order to standardize moisture levels across treatments.
28 Prior to behavioural trials, slugs ($n = 15$ individuals of each species) were selected randomly
29 and isolated in separate containers for two hours. The test slugs were then placed into arenas
30 along the dividing line between the beetle-exposed and control sections of paper. The initial
31 north-south orientation of each slug was consistent at the beginning of all trials but the left-
32 right positions of paper sections were switched before the next trial. The response of
33 individual slugs to residual scents from *P. niger* was never tested more than once. Slug
34 behaviour was recorded in trials of 2 hours duration using EthoVision® XT10 tracking

1 software. Beetle-exposed and test paper sections were defined in-program as zones, and slug
2 activity was measured as the cumulative duration (in seconds) and the velocity of movement
3 (cm/second) of individual slugs in each zone.

4
5

6 (2) *Slug secondary defence mechanisms*

7 *Collection and elemental analysis of trail mucus:*

8 Slug mucus may differ chemically among species (Cook, 1987; Skingsley *et al.*, 2000) and
9 depending on the part of the body from which it is collected (Skingsley, 2010). Therefore,
10 only trail mucus was examined from all three species for this study. Mucus samples were
11 collected from slugs before and after exposure to a stimulated attack (referred to hereafter as
12 non-stressed and stressed mucus, respectively).

13 Non-stressed mucus was collected by allowing slugs ($n = 20$ individuals per species) to crawl
14 into and within sterile glass petri dishes (diameter: 9cm). After sufficient quantities (mean
15 weight \pm SE = 5.1 ± 2.0 mg) of mucus had been secreted on the floor of the petri dish
16 (typically after a duration of 5-10 minutes), the slug was enticed out of the petri dish by
17 presenting a chopped carrot outside the dish (slugs were not removed by hand in case this
18 stimulated a stress response).

19 To prepare stressed mucus samples, the same individuals were placed in sterile glass petri
20 dishes 24 hours later and beetle attack was simulated by firmly pinching the mid-section and
21 tail of the slug repeatedly with a forceps without breaking the skin (after: Pakarinen, 1992).
22 This resulted in increased crawling speed and the production of highly viscous mucus in all
23 three slug species studied. Both non-stressed and stressed mucus preparations were scraped
24 from the petri dishes using a plastic straw and transferred into 0.5ml Eppendorf tubes.
25 Samples were freeze-dried to remove as much liquid water as possible (molluscan mucus is
26 composed of up to 80-99% water: Davies and Hawkins, 1998), so that they could be gold-
27 coated for SEM imaging, which is required prior to EDX analysis (discussed below). Slugs
28 and their mucus samples were weighed before and after simulated attack events. Mucus
29 samples were further weighed after freeze-drying to estimate the percentage water content
30 from the mucus of each species.

31 To investigate whether the elemental composition of mucus differs among species and
32 between stressed and non-stressed animals, mucus samples were imaged under scanning
33 electron microscopy and analysed using energy dispersive x-ray (SEM-EDX) spectroscopy.
34 Samples were imaged under high (x300) magnification with acceleration voltage set to 15kV.

1 EDX spectra were measured from three points of each mucus sample using INCA software
2 (Oxford). Means of these three spectra were used as an average elemental profile for each
3 mucus sample per individual. The reflectance of gold (Au) was removed and the percentage
4 weights of each element per sample were calculated in-program.

5

6 *Effect of calcium and mucus viscosity on carabid feeding behaviour:*

7 Test beetles were isolated and starved for 48hr prior to feeding trials. The sex of each beetle
8 was noted and its weight was recorded. Individuals were then given a choice between cat
9 food pellets (mean weight: $0.21\text{g} \pm 0.03\text{g}$) coated in a solution of distilled water and calcium
10 (99% granular: Sigma) or cat food pellets coated with distilled water. The concentration of
11 the calcium/water solution was selected based on the mean percentage weights of calcium
12 obtained from EDX analysis of stressed mucus (3g Ca per 100ml distilled water). Feeding
13 trials ($n = 15$) were carried out over two hours using a mix of male and female *P. niger*.
14 Beetle behaviour was recorded using Ethovision® XT10 software. The trials were conducted
15 in darkness during the beetle nocturnal phase in plastic boxes (15cm × 15cm × 25cm), the
16 floor of which was lined with damp white chromatography paper to maximise detection of
17 beetles by the tracking software. Each food treatment was defined in Ethovision® XT10 as a
18 zone, and feeding behaviour was assessed by beetle activity in each zone (i.e. the time spent
19 in contact and number of interactions with each food treatment).

20 It was observed that all three slug species exuded highly viscous mucus during the collection
21 of stressed mucus samples for elemental analysis. We therefore carried out additional trials to
22 test for possible effects of mucus viscosity on *P. niger* feeding behaviour. Artificial slug
23 mucus was created using odourless, non-toxic Xanthan gum powder (Freee Foods, UK) to
24 provide relatively low and high viscosity treatments. Cat food pellets (mean weight: $0.20\text{g} \pm$
25 0.02g) were then coated with one of two treatments representing different mucus viscosities:
26 low viscosity (0.5g Xanthan gum powder / 100mL distilled water), representing 'normal'
27 mucus, and high viscosity (2g Xanthan gum powder / 100mL distilled water), representing
28 'stressed' mucus. Both food treatments were offered to a mix of male and female *P. niger*
29 individuals in $n = 15$ choice trials which were also recorded and tracked in darkness during
30 the animals nocturnal phase using Ethovision® XT10 software, with each food treatment
31 defined as a zone, and feeding behaviour assessed by beetle activity (time spent in contact
32 and number of interactions with each food treatment) in each zone.

33

34 *Innate escape responses of slugs:*

1 The innate escape behaviour of each slug species was studied as an additional measure of a
 2 possible secondary defence mechanism. Individual slugs of each species (*D. reticulatum* $n =$
 3 58; *G. maculosus* $n = 108$; *L. marginata* $n = 66$) were shaken in-hand for 5 seconds and then
 4 placed into the centre of a petri dish (diameter: 9cm). The duration taken to escape (i.e. to
 5 crawl completely out of the petri dish) was recorded (in seconds) with a stopwatch. Each slug
 6 species possesses specific startle responses, and the expression of these behavioural responses
 7 was recorded after the individuals were placed into the petri dish: *D. reticulatum* exhibits a
 8 tail-wagging behaviour, whereas *G. maculosus* and *L. marginata* each exhibit different
 9 degrees of conglobation (curling up into a ball); with *G. maculosus* bending its sole
 10 completely in half to form a tight ball shape, and *L. marginata* bending its sole to form a ‘c-
 11 shape’ (Rowson *et al.*, 2014).

12

13 (3) *Predation tactics of P. niger in no-choice feeding trials*

14 *Pterostichus niger* beetles used in no-choice feeding trials were maintained in the same
 15 conditions as those used to examine the defensive mode of slug mucus described above.
 16 Beetles were starved for one week prior to no-choice feeding trials (Symondson *et al.*, 1997)
 17 and then placed into a Parafilm®-sealed petri dish (diameter: 9cm) with a single individual of
 18 either *D. reticulatum* ($n = 20$), *G. maculosus* ($n = 20$), or *L. marginata* ($n = 20$). The
 19 behaviour of $n = 60$ *P. niger* individuals (33 females, 27 males) and their potential slug prey
 20 was recorded in darkness for 24hours. Video files were analysed manually by point-sampling
 21 every 2 minutes and recording the behavioural states of carabids and slugs as well as specific
 22 behavioural events (mantle attacks, tail attacks and aversions) occurring within a 1-minute
 23 period following the point-sample (Table 1). Beetles and slugs were weighed immediately
 24 prior to each feeding trial. After each trial had concluded, beetles were re-weighed and it was
 25 noted whether or not slugs had been eaten. No-choice feeding trials were carried out from
 26 June to August 2016.

27

Table 1. Descriptions of carabid and slug behavioural categories observed in no-choice feeding trials

Carabid behaviour	Description
Mantle attack	<i>P. niger</i> visibly biting the mantle or head-region of a slug
Tail attack	<i>P. niger</i> visibly biting distally from the mantle to the sides and tip of a slugs tail
Aversion	<i>P. niger</i> cleaning mucus from mandibles, antennae or limb-joints following a mantle or tail attack
Slug behaviour	Description
Hunched	Slug immobile in a hunched, bell-shaped posture: tentacles retracted beneath mantle
Moving	Slug actively crawling

Not Moving
Dead

Slug body extended but immobile
Slug dead; immobile with mouthparts distended and/or slug laying laterally

1
2 *Statistical Analysis*
3 Paired t-tests were used to examine whether slug behaviour (mean time and mean velocity of
4 movement) differed significantly between beetle-exposed and control zones. A one-way
5 ANOVA was used to test whether these behavioural parameters differed significantly among
6 slug species in both beetle-exposed and control zones. A Tukey post-hoc tests were
7 subsequently used to examine whether each pair-wise combination of slug species differed
8 significantly in mean time or mean movement velocity in beetle-exposed zones. A one-way
9 ANOVA was used to test for significant species differences in the mean percentage water
10 content from both non-stressed and stressed mucus samples. Paired t-tests were then used to
11 examine if the mean percentage water content differed significantly between non-stressed and
12 stressed mucus samples for each species. Paired t-tests were also used to examine whether the
13 mean percentage weight of each element detected via EDX differed significantly between
14 non-stressed and stressed mucus samples from each slug species. Some elements were not
15 normally distributed in mucus samples for each species (Supplementary Table A2) and these
16 were instead compared using Wilcoxon's sign-rank test. One-way ANOVAs were used to test
17 whether the mean percentage weights of calcium differed significantly among slug species in
18 both non-stressed and stressed mucus. Pearson's correlation was used to test whether there
19 was a significant relationship between slug weight and the percentage weights of calcium
20 expressed in stressed mucus samples.
21 Potential differences in feeding behaviour (i.e. time in contact with, and number of
22 interactions with food treatments) between male and female *P. niger* beetles were examined
23 using a Mann-Whitney U-test. When no significant difference was observed between male
24 and female *P. niger* beetles, data were pooled to examine whether feeding behaviour differed
25 significantly between calcium-treated and control food treatments; and between low- and
26 high-viscosity food treatments, also using Mann-Whitney's U-tests.
27 Species differences in mean escape times were examined using a one-way ANOVA and
28 Tukey's post-hoc tests were performed on each pair-wise species combination. Pearson's
29 correlation was used to test for significant relationships between slug weights and escape
30 times.
31 Binary logistic regression was performed to assess the impact of multiple factors on the
32 likelihood of predation occurring in $n = 60$ no-choice feeding trials. The model contained

1 seven independent variables (the frequency of tail attacks per 24 hour predation trial, the
2 frequency of mantle attacks per 24 hour predation trial, the weight difference between beetles
3 and slugs per trial, the species identity of slugs, and the sex of each beetle).
4 Data from trials where successful predation occurred (i.e. $n = 25$ trials where slugs were
5 successfully killed and consumed) were subsequently pooled to characterize the general
6 predation behaviour of *P. niger* in further detail. A General Linear Model (GLM) was
7 performed to assess the impacts of multiple independent variables on the duration taken until
8 a slug was killed and consumed by *P. niger*. The GLM examined the effects of the frequency
9 of tail and mantle attacks, the species identity of the slugs, and the sex of the beetles on the
10 duration taken until a slug was killed and consumed. The interaction between the sex of *P.*
11 *niger* and the species identity of the slugs was included in the GLM.
12 Spearman's correlation was used to test whether the frequency of mantle and tail attacks
13 (both normally distributed, continuous variables) were significantly associated with the
14 duration taken until a slug died (an ordinal variable). Pearson's correlation was used to
15 examine whether tail and mantle attacks were significantly correlated with the numbers of
16 aversive behavioural responses. A Student's t-test was used to test whether *P. niger* differed
17 significantly in the mean frequency of tail or mantle attacks performed on slugs.

18

19 **Results**

20 (1) *Slug primary defence mechanisms*

21 All slug species spent significantly less time and exhibited significantly greater velocities
22 (cm/second) in areas exposed to *P. niger* compared to control areas (Table 2). There was no
23 significant difference among slug species in the mean time spent in control zones ($F(2, 42) =$
24 $2.432, P = 0.10$), or in mean movement velocities in control zones ($F(2, 42) = 2.387, P =$
25 0.104). Mean time spent in beetle-exposed zones did not differ significantly among species
26 ($F(2, 42) = 2.527, P = 0.092$). However, mean movement velocities in beetle-exposed zones
27 did differ significantly among species ($F(2, 42) = 4.95, P = 0.012$). A Tukey post-hoc test
28 revealed that *D. reticulatum* velocity in beetle-exposed zones was significantly greater than
29 that of *L. marginata* ($P = 0.009$) but there were no significant differences in mean velocity
30 between *D. reticulatum* and *G. maculosus* ($P = 0.368$), or between *G. maculosus* and *L.*
31 *marginata* ($P = 0.191$) in beetle-exposed zones.

Table 2. Behaviour of each slug species in zones exposed to *P. niger* or control zones (s = seconds)

Species	Beetle-exposed		Control		Mean Diff.	t (14)	P
	Mean	SD	Mean	SD			

Time in zone (s)	<i>D. reticulatum</i>	1937.13	1163.90	5262.87	1163.90	-3325.73	-5.53	<0.001
	<i>G. maculosus</i>	2502.53	1139.56	4644.00	1144.19	-2141.47	-3.64	0.003
	<i>L. marginata</i>	2874.73	1212.45	4317.80	1199.91	-1443.07	-2.32	0.036
Velocity in zone (cm/s)	<i>D. reticulatum</i>	0.09	0.01	0.06	0.02	0.03	4.75	<0.001
	<i>G. maculosus</i>	0.08	0.02	0.05	0.02	0.03	3.35	0.005
	<i>L. marginata</i>	0.07	0.02	0.04	0.01	0.04	4.92	<0.001

1 (2) *Slug secondary defence mechanisms*

2 *Elemental analysis of trail mucus:*

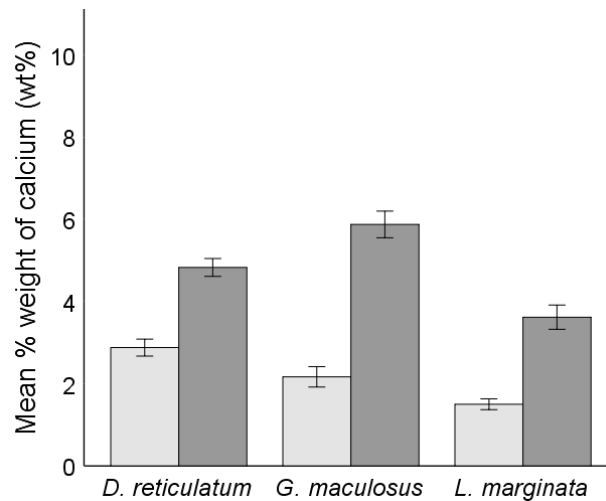
3 There was no significant difference among the slug species studied in mean percentage water
4 content for either non-stressed or stressed mucus (Appendix: Table A1). In addition, there
5 was no significant difference in mean percentage water content between non-stressed and
6 stressed mucus for each slug species (Appendix: Table A2).

7 In total, 10 elements were detected from both stressed and non-stressed mucus samples of all
8 three slug species studied (Appendix: Table A2). Attack stimulus resulted in a significant
9 increase in the mean percentage weight of calcium in stressed mucus of all three slug species
10 (Fig. 1). The mean percentage weights of calcium in stressed mucus samples differed
11 significantly among slug species ($F(2, 57) = 16.01, P < 0.001$). Tukey post-hoc test revealed
12 that stressed mucus of *G. maculosus* contained significantly greater mean percentage weights
13 of calcium than both *D. reticulatum* ($P = 0.03$) and *L. marginata* ($P < 0.01$); and that stressed
14 mucus of *D. reticulatum* contained significantly greater mean percentage weights of calcium
15 than *L. marginata* ($P = 0.01$).

16 There was no significant correlation between slug weight and the percentage weights of
17 calcium recorded in mucus samples from stressed individuals ($r = 0.196, P = 0.133$). The
18 mean percentage weights of carbon and oxygen were also greater in stressed mucus samples
19 for *D. reticulatum* and *G. maculosus* (Appendix: Table A2); and were significantly greater
20 for *L. marginata* (carbon: $t(19) = -2.323, P = 0.031$; oxygen: $t(19) = -2.351, P = 0.029$).

21 There were no significant differences in any other elements between non-stressed and
22 stressed mucus samples for any of the slug species studied (Appendix: Table A2).

23



1
2 Figure 1. The percentage weights of calcium were significantly greater in stressed mucus
3 (dark grey bars) for all three slug species studied (*D. reticulatum*: $t(19) = -7.571$, $P < 0.001$;
4 *G. maculosus*: $t(19) = -9.724$, $P < 0.001$; *L. marginata*: $t(19) = -7.361$, $P < 0.001$). Bars show
5 mean percentage weights \pm SE
6

7 *Effect of calcium and viscosity on beetle feeding behaviour:*

8 Data from female and male *P. niger* were pooled since no significant difference was detected
9 between male and female *P. niger* feeding behaviour in calcium-treatment or mucus viscosity
10 trials (i.e. frequency of interactions: $U = 104.5$, $P = 0.759$; $U = 87.5$, $P = 0.313$ respectively
11 and time spent interacting with each food treatment: $U = 101$, $P = 0.667$; $U = 98.5$, $P = 0.580$
12 respectively).

13 Beetles visited food treated with low viscosity gel significantly more often and spent
14 significantly more time feeding than food treated with high viscosity gel (Table 3). No
15 significant difference was found, however, in beetle feeding behaviour (frequency of visits
16 and time spent interacting with food) between food treated with 3% calcium and food treated
17 with distilled water.
18

Table 3. Effects of calcium and gel viscosity on beetle feeding behaviour. Median frequencies of interaction, and median times interacting with either treatment per 2 hour trial are shown with inter-quartile ranges presented in brackets. Results from significance tests (Mann Whitney U) are shown between food treatments (s = seconds)

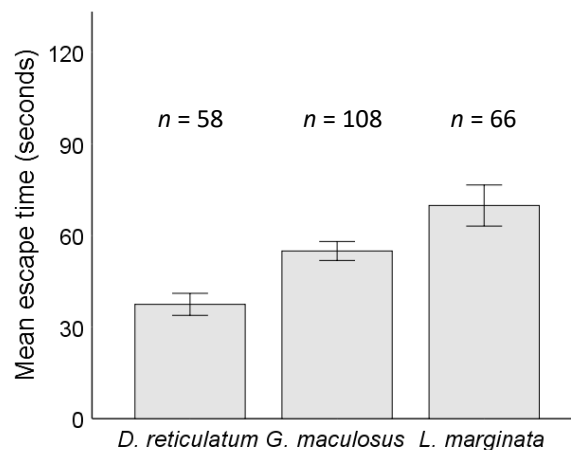
<i>Effect of calcium</i>	Distilled water	3% calcium solution	Mann-Whitney U	<i>P</i>
Median frequency of interactions	25.50 (23)	31.50 (35)	104.5	0.744
Median time spent interacting (s)	1636.50 (1085)	1980.00 (2162)	106.5	0.806
<i>Effect of viscosity</i>	0.5% gel	2% gel	Mann-Whitney U	<i>P</i>
Median frequency of interactions	36.00 (40)	11.00 (23)	53.0	0.013
Median time spent interacting (s)	1241.00 (2109)	88.00 (935)	56.5	0.019

1 *Innate escape responses of slugs:*

2 Each slug species differed significantly in the time taken to escape from a petri dish ($F(2,$
3 $229) = 10.61, P < 0.001$; Fig. 2) following handling for five seconds. A Tukey post-hoc test
4 revealed that *D. reticulatum* exhibited a faster mean escape time than both *G. maculosus* ($P =$
5 0.018) and *L. marginata* ($P < 0.001$), and that *G. maculosus* exhibited a faster mean escape
6 time than *L. marginata* ($P = 0.041$).

7 A significant but weak negative correlation was found between escape time and slug weight
8 for *L. marginata* ($r = -0.266, P = 0.031$). Escape time was not significantly correlated with
9 weight for either *D. reticulatum* ($r = 0.255, P = 0.053$) or *G. maculosus* ($r = 0.014, P =$
10 0.887). Intra-specific behavioural differences in escape response were observed in *D.*

11 *reticulatum*, with 24 out of 58 individuals (41%) exhibiting a ‘tail-wagging’ response prior to
12 escape. Conglobation (curling into a ball or ‘c-shape’) was observed prior to escape in 100%
13 of *G. maculosus* and *L. marginata* individuals tested.



14
15 Figure 2. Each slug species differed significantly in the duration taken to escape after
16 handling. Bars show mean escape times \pm SE
17

18 (3) *Predation tactics of Pterostichus niger in no-choice feeding trials*

19 The null model containing all predictors was statistically significant, $\chi^2(6, n = 60) = 51.90, P$
20 < 0.001 , indicating that the model successfully distinguished between trials where slugs were
21 and were not killed and eaten by *P. niger*. The model explained between 58% (Cox and Snell
22 R square) and 78% (Nagelkerke R square) of the variance in predation success, and correctly
23 classified 91.7% of cases. Three of the independent variables made a significant contribution
24 to the model (the frequency of tail attacks per 24 hour predation trial [$P = 0.004$], the
25 frequency of mantle attacks per 24 hour predation trial [$P = 0.003$], and the species identity of
26 slugs [*D. reticulatum*: $P = 0.007$; *G. maculosus*: $P = 0.025$; *L. marginata*: $P = 0.002$]; Table

1 4). The frequency of tail and mantle attacks were both the strongest predictors of predation
 2 success, recording almost equal odds ratios (tail attacks: 1.06; mantle attacks: 1.09).

3

4 Table 4. Logistic regression predicting the likelihood of successful predation by *P. niger*.

<u>Independent variables</u>	B	SE	Wald	df	P	Odds Ratio	95% CI for Odds ratio	
							<u>Lower</u>	<u>Upper</u>
Tail attacks	0.06	0.02	8.15	1	0.004	1.06	1.02	1.11
Mantle attacks	0.09	0.03	8.64	1	0.003	1.09	1.03	1.16
Weight difference	-1.20	1.16	1.09	1	0.297	0.30	0.03	0.29
<i>D. reticulatum</i>			9.83	2	0.007			
<i>G. maculosus</i> *	-4.64	2.07	5.02	1	0.025	0.01	0.00	0.56
<i>L. marginata</i> *	-8.52	2.73	9.73	1	0.002	0.00	0.00	0.04
Sex of beetle	-0.22	1.22	0.03	1	0.855	0.80	0.07	8.77
Constant	-4.58	1.64	7.80	1	0.005	0.01		

5 *The coefficients for *G. maculosus* and *L. marginata* are set relative to the effects of *D. reticulatum*

6

7 Of the variables examined in GLM analysis, the frequency of mantle attacks was the only
 8 significant predictor of the time taken for a prey slug to be killed and consumed by *P. niger*
 9 ($P = 0.001$). Species identity of the slugs was marginally non-significant ($P = 0.063$; Table
 10 5).

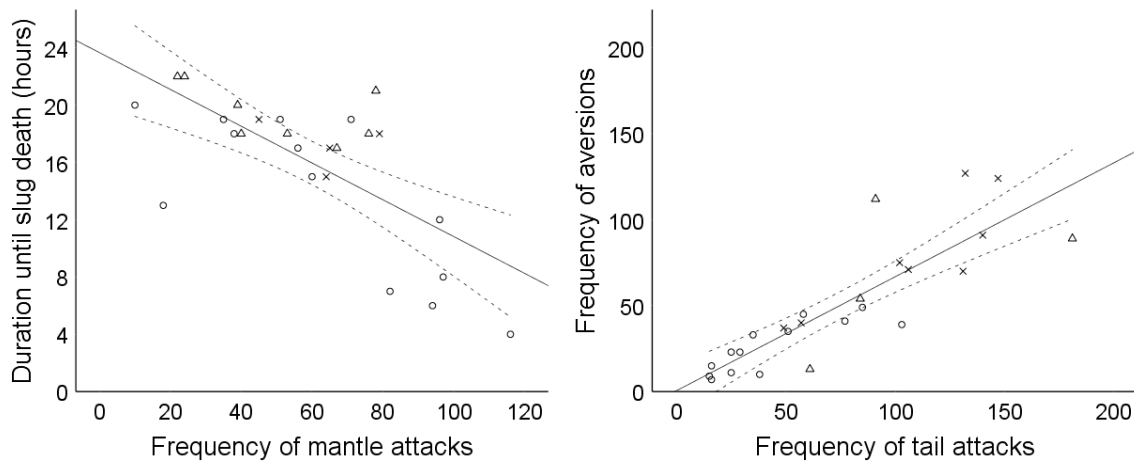
11

12 Table 5. General Linear Model assessing the contribution of multiple factors to the duration
 13 taken until slugs were successfully killed and consumed by *P. niger*.

	Sum of squares	df	Mean square	F	P
<u>Main effects</u>					
Tail attacks	4.36	1	4.36	0.53	0.477
Mantle attacks	146.58	1	146.58	17.75	0.001
Sex of beetle	5.15	1	5.15	0.62	0.440
Slug species	53.61	2	26.81	3.25	0.063
<u>Interaction terms</u>					
Sex of beetle × slug species	18.79	1	18.79	2.28	0.149

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The frequency of mantle attacks was significantly and negatively correlated with the time taken for a prey slug to be killed (Spearman's $r = -0.633$, $P < 0.01$; Fig. 3a). There was a significant and positive correlation between tail attacks by *P. niger* and time until death for slugs across each successful feeding trial (Spearman's $r = 0.697$, $P < 0.01$). The frequency of tail attacks was also significantly and positively correlated with the frequency of aversive behavioural events displayed by *P. niger* (Pearson's $r = 0.855$, $P < 0.01$; Fig. 3b). There was no significant correlation between the frequency of mantle attacks and aversive behavioural events (Pearson's $r = -0.354$, $P = 0.083$). There was no significant difference between the mean number of mantle attacks and tail attacks performed by *P. niger* over $n = 25$ feeding trials where predation was successful ($t = 1.395$, $P = 0.171$).



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Figure 3 (a) The frequency of mantle attacks performed on slugs by *Pterostichus niger* was negatively correlated with the time taken until death for slugs; and (b) the frequency of tail attacks by *Pterostichus niger* was positively correlated with the number of aversive behavioural events in no-choice feeding trials where predation was successful. Dotted lines = 95% CI; Symbols: o = *Deroceras reticulatum*; x = *Geomalacus maculosus*; Δ = *Lehmanna marginata*

20 Discussion

21 Primary defence mechanisms

22 All three slug species spent less time and exhibited greater velocities in areas exposed to *P. niger*. This is in agreement with previous studies which have demonstrated that the behaviour of *D. reticulatum* is modified in the presence of residual scents from carabid beetles (Armsworth *et al.*, 2005; Bursztyka *et al.*, 2013; Bursztyka *et al.*, 2016), but the current study is the first demonstration that this primary defence mechanism is a common response in terrestrial slugs. Anti-predator behaviour in prey species may not be expected to evolve in response to the presumably weak selective pressure imposed by generalist predators unless

1 the prey class constitutes a major proportion of the diet of a predator species (Armsworth *et*
2 *al.*, 2005). Since avoidance of zones where *P. niger* had recently visited was a shared
3 response among all slug species examined, this suggests that terrestrial slugs form a major
4 component of the diet of many carabid species generally. Recent work has pointed to the
5 seasonal importance of terrestrial slugs as a food source for generalist carabids during periods
6 when alternative prey is unavailable but when slugs occur at high densities, such as in
7 Autumn and early Spring (Jelaska *et al.*, 2014). Generalist ground beetles could therefore be
8 considered a trophic species (Sih *et al.*, 1998) insofar as multiple carabid species may elicit
9 similar behavioural responses in multiple slug species, which may have led to the evolution
10 of general avoidance behaviour observed in each slug species in this study. *Deroceras*
11 *reticulatum* appeared to show the strongest avoidance of *P. niger* (i.e. spent the least amount
12 of time and exhibited the greatest velocity in beetle-exposed zones), followed by *G.*
13 *maculosus* and *L. marginata*, in turn. This may reflect the average exposure of *D. reticulatum*
14 to predation by multiple generalist carabids in open habitats, relative to *G. maculosus*, a
15 habitat generalist which occurs in both open habitats and forested areas, and *L. marginata*
16 which is an arboreal forest specialist.

17

18 *Secondary defence mechanisms*

19 The importance of slug mucus as a defence against generalist predators such as carabids has
20 been examined in multiple studies to date (Pakarinen, 1994; Mair and Port, 2002; Foltan,
21 2004) but the current study is the first demonstration that terrestrial slug mucus changes
22 chemically after exposure to predation and highlights the apparent important contribution of
23 calcium secretion as a secondary defence mechanism which is common to slugs. Simulated
24 predation resulted in a significant increase in the percentage weight of calcium measured
25 from the mucus of all three slug species studied. The percentage weights of calcium differed
26 significantly among slug species in both non-stressed and stressed samples, despite slug size
27 showing no correlation with the concentrations of calcium in stressed mucus samples. This
28 suggests that defensive capabilities differ with slug species to some extent, as had been
29 previously suggested (Rollo and Wellington, 1979; Pakarinen, 1994; Foltan, 2004), but that
30 the general anti-predator response (i.e. elevated levels of calcium secretion and increased
31 mucus viscosity) is probably consistent across terrestrial slugs in general. Mucus production
32 is energetically expensive (Denny, 1980) and, given that calcium is a vital element for
33 molluscs (Fournié and Chétail, 1984), it follows that slugs should possess a behavioural
34 mechanism to regulate calcium secretion. It is possible that mechanical stimulation causes

1 calcium glands in the slug epithelium to rupture and release calcium into a defensive mucus
2 cocktail in response to predator attack. Dreyup-Olsen and Martin (1982) demonstrated that
3 mechanical stimulation resulted in increased calcium concentrations in sac preparations of
4 slug body wall, on which basis they suggested that slugs possess a calcium secretory process.
5 Calcium release was a general response to simulated predation attempts in all three slug
6 species in this study and there are many previous studies which suggest that this may be an
7 important secondary defence mechanism common to many terrestrial molluscs. Dexheimer
8 (1951) recorded two mucus consistencies in the snail *Helix pomatia* Linnaeus, which
9 appeared to be dependent on the behavioural state of the animal: a clear secretion was
10 produced under normal conditions whereas irritated snails produced more viscous, 'creamy'
11 secretions. Champion (1961) similarly noted that the mucus of the snail *Cornu aspersum*
12 Müller (= *Helix aspersa*) became more opaque and 'milky' when the animal was stressed,
13 possibly due to calcium secretions which also increased mucus viscosity, and suggested a
14 defensive role for this change in mucus consistency. In this study, calcium alone did not
15 negatively impact on the feeding behaviour of *P. niger*; rather, levels of viscosity appeared to
16 be the determining factor in deterring beetle feeding activity. The defensive properties of slug
17 mucus therefore appear to be enhanced in response to predator attack, such that the calcium
18 content of mucus rapidly increases when slugs are exposed to periods of acute stress (e.g.
19 predation attempts). The elevated level of calcium in slug mucus post-attack probably
20 increases the viscosity of mucus. Braun *et al.* (2013) demonstrated the importance of calcium
21 in determining mucus viscosity from the slug *Arion subfuscus* Draparnaud, and the results
22 from this current study demonstrate that calcium levels in mucus are elevated when slugs are
23 exposed to mechanical stimulation which imitates predator attack.

24 Although all slug species examined in this study exuded significantly greater percentage
25 weights of calcium when stressed, *G. maculosus* exuded the greatest percentage weight of
26 calcium in stressed mucus samples. This may reflect the likely greater diversity of potential
27 predators faced by *G. maculosus*, which lives in both open and forested habitats. It is known
28 from other taxa, for example, that predation from multiple sources can impose stronger
29 selection for antipredator behaviour than would be expected from a single predator class (e.g.
30 in birds: Morosinotto *et al.* [2010]; in fish: Templeton and Shriner [2004]). Alternatively, the
31 high percentage weights of calcium recorded in stressed *G. maculosus* mucus could be related
32 to skin thickness. As a medium-large sized arionid slug, *G. maculosus* possesses thicker skin
33 than limacid (e.g. *L. marginata*) or agriolimacid (e.g. *D. reticulatum*) slugs, and may
34 therefore possess a relatively higher proportion of epithelial mucus and calcium glands.

1 Pakarinen (1994) and Foltan (2004) both found that generalist carabids (*P. niger* and *P.*
2 *melanarius*, respectively) preferred slug species which possess thin skin. Pakarinen (1994)
3 found that *P. niger* predation was successful on the thick-skinned arionid slug *Arion fasciatus*
4 Nilsson only once the mucus supply of the slug had been exhausted after prolonged exposure
5 to a stressful stimulus. Species differences in calcium secretion may therefore afford slugs
6 different degrees of protection against predators and ultimately influence prey choice by
7 generalist predators in the field.

8 Two other elements, carbon and oxygen, which were detected in all mucus samples, were in
9 greater concentrations in stressed mucus samples for all slug species, and were significantly
10 greater for *L. marginata*. This may correspond to the production and secretion of species-
11 specific molecules signalling stress, which conspecific slugs have been shown to avoid
12 (Pakarinen, 1992). It is also possible that the increases in carbon and oxygen are linked to the
13 significant increase in calcium for all slug species examined through the secretion of calcium
14 carbonate granules, which Champion (1961) attributed to the white colour of irritated mucus
15 from the snail *Cornu aspersum*.

16 Escape times also varied significantly among species, with *D. reticulatum* possessing the
17 fastest escape time, followed by *G. maculosus* and *L. marginata*, respectively. Species-
18 specific escape responses were also recorded in each slug species. Intra-specific behavioural
19 variation was observed in *D. reticulatum*, in which a ‘tail-wagging’ response was recorded in
20 41% of the slugs examined. Rollo and Wellington (1979) found that tail-wagging in *D.*
21 *reticulatum* was an effective strategy in defending against attacks from con- and
22 heterospecific slugs, which would retreat after receiving a blow from the tail. Tail-wagging
23 may have a similar effect on predaceous carabids and function by focusing predator attention
24 towards the tail region of the slug, thereby limiting the amount of predator attacks targeted at
25 the vulnerable mantle region. Conglobation was a response recorded in 100% of all *G.*
26 *maculosus* and *L. marginata* individuals examined. Conglobation might be an adaptation to
27 avoid predation by birds, as it is only expressed when the slugs are removed from their
28 substrate. Additionally, *G. maculosus* possesses background matching camouflage (O’Hanlon
29 *et al.*, 2017) and *L. marginata* also closely resembles the visual properties of tree trunks (i.e.
30 it is brown with dark lateral bands and mottling) which is consistent with evolution by
31 selection from visual predators such as birds. This might also explain the slow escape times
32 of these two species relative to *D. reticulatum* in that slow-moving, camouflaged prey should
33 be less detectable than conspicuous highly mobile alternative prey (Hall *et al.*, 2013).

34

1 *Predation tactics of Pterostichus niger in no-choice feeding trials*

2 In no-choice feeding trials, the frequency of mantle attacks was the only variable examined
3 which significantly predicted the duration taken for a prey slug to be killed and consumed by
4 *P. niger*. Furthermore, the frequency of mantle attacks was strongly and negatively correlated
5 with the time it took a slug to be killed. Carabid beetles which are specialist gastropod-
6 feeders are capable of overcoming the defensive mucus of even large, thick-skinned arionid
7 slugs. Pakarinen (1994) noted that *Carabus violaceus* Linnaeus and *Cychrus caraboides*
8 Linnaeus, both of which were described as mollusc-specialists, prevented the slug *A.*
9 *fasciatus* from exuding defensive mucus by stabbing the mantle region of the slug with their
10 mandibles, which may paralyze the slugs either by delivering a toxin or by mechanically
11 destroying the cerebral ganglia. Larvae of the slug-killing fly *Tetanocera elata* Fabricius also
12 appear to feed most often at the head-region of slugs, or by burrowing beneath the slug
13 mantle (Knutson *et al.*, 1965, Hynes *et al.*, 2014) and may additionally inject an immobilizing
14 toxin (Knutson and Vala, 2011). Knutson *et al.* (1965) also reported that the defensive mucus
15 exuded by *Deroceras* (= *Agriolimax*) slugs did not deter attack from the malacophagous fly
16 larvae. Pakarinen (1994) found that the generalist carabid *P. niger* did not target a specific
17 region of the body of the slug, and the results from the present study similarly found that *P.*
18 *niger* did not show a preference for attacking either the mantle or tail region of a slug. The
19 frequency of tail attacks was positively correlated with the number of aversions displayed by
20 *P. niger* in feeding trials, which is probably due to the production of defensive mucus by the
21 slugs. This indicates that the defensive mucus of slugs may be an adaptation to predation
22 attempts from multiple generalist predators but not to malacophagous specialists. Many
23 carabid beetles which prey upon slugs also feed on a host of other invertebrate species such
24 as earthworms, lepidopteran larvae and woodlice (Jelaska *et al.*, 2014). The release of highly
25 viscous mucus may afford attacked slugs sufficient time to escape by causing attacking
26 carabids to increase the frequency of non-predatory behaviour (e.g. cleaning), and may
27 therefore represent a useful strategy to avoid predation by generalists which could
28 subsequently switch their attention to alternative prey.

29 The species identity of prey slugs was a significant predictor of predation success in no-
30 choice feeding trials. *Deroceras reticulatum* was the most frequently killed slug species in
31 no-choice feeding trials and was successfully killed and eaten by *P. niger* in 60% of these
32 trials. It is interesting that, despite their relatively low calcium secretions and slow escape
33 responses, *L. marginata* individuals were eaten least often (in only 20% of trials) by *P. niger*.
34 Anti-predator adaptations which are costly or are no longer beneficial are predicted to be lost

1 rapidly following isolation (Blumstein, 2006). The relatively weak expression of primary and
2 secondary defence mechanisms observed may therefore reflect the likely low encounter rates
3 of the arboreal *L. marginata* with ground beetles in forested habitats. These results are
4 broadly in agreement with recent experimental work by Saeki *et al.* (2017) who demonstrated
5 that seasonal migration into tree canopies by the land snail *Euhadra brandtii sapporo* Ijima is
6 directly influenced by ground beetle predation. Intense selection by ground beetles in the past
7 may have similarly contributed to the evolution of arboreality in *L. marginata* and *G.*
8 *maculosus*. Alternatively, it is possible that *L. marginata* may contain toxins which are
9 noxious to carabids, as was suggested for *Tandonia budapestensis* Hazay by Symondson
10 (1997).

11 12 *Conclusions*

13 The results of this study indicate that different slug species possess common primary and
14 secondary anti-predator defences. However, the expression of these defence mechanisms
15 varies among species. This appears to reflect the ecology of each slug species examined: *D.*
16 *reticulatum*, as a numerically dominant open habitat-specialist probably encounters carabids
17 frequently and, as such, it appears to possess the strongest primary defence, and the fastest
18 escape time. *Lehmannia marginata*, as an arboreal forest specialist, likely encounters
19 predation from ground beetles only relatively rarely. *Geolamachus maculosus*, as a niche
20 generalist, is likely exposed to a greater number of potential predators from both open and
21 forested habitats, explaining its intermediate primary and secondary defences. Highly
22 viscous, calcium-enriched mucus secretions from attacked *G. maculosus* may be reflective of
23 a greater diversity of potential predator species encountered in both open and forested
24 habitats.

25 Most of our knowledge about predator-prey evolutionary ecology comes from considering
26 single species pairings. Although the common secondary and primary responses observed in
27 this study can be explained as adaptations to avoid a single trophic species (generalist
28 carabids), species-specific primary (camouflage) and secondary (conglobation) defence
29 mechanisms of both *G. maculosus* and *L. marginata* cannot be satisfactorily explained by
30 considering a single predator class, and instead point to the role of visual predators such as
31 birds. This highlights the importance of considering predation from multiple sources when
32 investigating anti-predator adaptations, and adds to the growing body of evidence that
33 multiple predators have effects on the expression of behaviour, morphology and ecology of
34 species over evolutionary timescales.

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Appendix

Table A1 Results of one-way ANOVA tests comparing mean percentage water content in non-stressed and stressed mucus samples

Mucus treatment	Species	Mean % water content ± SD	Between-groups ANOVA	
			F(2, 57)	P
Non-stressed	<i>D. reticulatum</i>	89.82 ± 8.44	1.193	0.311
	<i>G. maculosus</i>	92.19 ± 4.96		
	<i>L. marginata</i>	88.95 ± 6.69		
Stressed	<i>D. reticulatum</i>	92.89 ± 7.56	0.301	0.741
	<i>G. maculosus</i>	91.36 ± 14.97		
	<i>L. marginata</i>	93.89 ± 6.84		

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Table A2 Results of hypothesis tests examining percentage water content and percentage weights of all elements before and after attack stimulus for all slug species.

	Species	Non-stressed	Stressed	Test statistic*	P
Mean % water content	<i>D. reticulatum</i>	89.82 ± 8.44	92.89 ± 7.56	t(19) = -1.09	0.287
	<i>G. maculosus</i>	92.19 ± 4.96	91.36 ± 14.97	t(19) = 0.22	0.830
	<i>L. marginata</i>	88.95 ± 6.69	93.89 ± 6.84	t(19) = -2.05	0.054
Mean % weight C	<i>D. reticulatum</i>	45.69 ± 5.44	47.52 ± 2.32	t(19) = -1.32	0.202
	<i>G. maculosus</i>	48.81 ± 3.86	49.88 ± 3.31	t(19) = -0.87	0.396
	<i>L. marginata</i>	46.68 ± 2.94	50.43 ± 5.74	t(19) = -2.32	0.031
Mean % weight O	<i>D. reticulatum</i>	35.74 ± 2.36	36.76 ± 3.89	t(19) = -0.96	0.348
	<i>G. maculosus</i>	36.07 ± 2.22	37.38 ± 4.29	t(19) = -1.20	0.244
	<i>L. marginata</i>	37.89 ± 3.20	39.63 ± 2.59	t(19) = -2.35	0.029
Mean % weight Na	<i>D. reticulatum</i>	1.96 ± 1.12	1.67 ± 0.76	t(19) = 1.06	0.303
	<i>G. maculosus</i>	0.51 ± 0.67	0.43 ± 0.76	Z = -0.39	0.695
	<i>L. marginata</i>	1.38 ± 0.94	1.05 ± 0.76	t(19) = 1.04	0.311
Mean % weight Mg	<i>D. reticulatum</i>	0.56 ± 0.73	0.54 ± 0.44	Z = -0.36	0.723
	<i>G. maculosus</i>	0.65 ± 0.38	0.65 ± 0.46	t(19) = -0.04	0.997
	<i>L. marginata</i>	0.81 ± 0.12	1.14 ± 0.09	Z = -1.76	0.079
Mean % weight Al	<i>D. reticulatum</i>	2.46 ± 2.75	1.59 ± 2.59	Z = -1.35	0.177
	<i>G. maculosus</i>	0.12 ± 0.06	undetected**	n/a**	n/a**
	<i>L. marginata</i>	0.54 ± 0.28	undetected**	n/a**	n/a**
Mean % weight P	<i>D. reticulatum</i>	undetected**	undetected**	n/a**	n/a**
	<i>G. maculosus</i>	1.84 ± 1.69	1.90 ± 1.41	t(19) =	0.893
	<i>L. marginata</i>	1.84 ± 1.51	1.09 ± 1.36	t(19) = 1.50	0.150
Mean % weight Cl	<i>D. reticulatum</i>	4.06 ± 1.09	4.13 ± 1.49	t(19) = -0.18	0.863
	<i>G. maculosus</i>	1.98 ± 1.48	2.04 ± 1.21	t(19) = -0.13	0.898
	<i>L. marginata</i>	2.66 ± 2.05	2.69 ± 1.49	t(19) = -0.05	0.960
Mean % weight K	<i>D. reticulatum</i>	4.81 ± 1.96	5.40 ± 1.23	t(19) = -1.29	0.212
	<i>G. maculosus</i>	2.95 ± 1.63	2.36 ± 1.04	t(19) = -0.14	0.893
	<i>L. marginata</i>	4.71 ± 1.43	4.86 ± 2.26	t(19) = -0.25	0.809
Mean % weight Ca	<i>D. reticulatum</i>	2.88 ± 0.92	4.83 ± 0.97	t(19) = -7.57	<0.001
	<i>G. maculosus</i>	2.17 ± 1.11	5.87 ± 1.45	t(19) = -9.72	<0.001
	<i>L. marginata</i>	1.49 ± 0.59	3.62 ± 1.32	t(19) = -7.36	<0.001
Mean % weight S	<i>D. reticulatum</i>	undetected**	undetected**	n/a**	n/a**
	<i>G. maculosus</i>	undetected**	undetected**	n/a**	n/a**
	<i>L. marginata</i> **	0.06 ± 0.06**	0.42 ± 0.16**	n/a**	n/a**

*paired t-test results are shown for before-after comparisons of mean values where data are normally distributed; Wilcoxon's rank test results are shown for before-after comparisons of non-normal data. Tests performed are denoted by t and Z where paired t-test or Wilcoxon's tests were used, respectively

** Significance test not performed due to element not being detected in either non-stressed or stressed mucus samples (or both), or detected only once (as in S for *L. marginata* non-stressed mucus)

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