Tolerance to extreme hot and cold temperatures in the EU protected terrestrial slug *Geomalacus maculosus* Allman, 1843 (Mollusca: Arionidae)

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Terrestrial molluscs are frequently exposed to a wide range of temperatures (Ansart & Vernon, 2003) but temperature tolerance studies have focussed mainly on land snails (Udaka, Goto & Numata, 2008; Slotsbo, Hansen & Holmstrup, 2011) with fewer studies on terrestrial slugs. The lack of external physical protection offered by a shell and epiphragm make slugs more sensitive to extreme heat or cold (Schweizer, Triebskorn & Köhler, 2019) as their body is in direct contact with the environmental temperatures. This, combined with the fact that their skin is covered in a layer of moisture providing mucus, makes them susceptible to inoculative freezing (Storey, Storey & Churchill, 2007; Udaka, Goto & Numata, 2008; Slotsbo, Hansen & Holmstrup, 2011; Slotsbo *et al.*, 2012), whereby contact with external ice can induce the formation of internal ice crystals thereby freezing slug tissues. Indeed, mucus secreted by the slugs can also undergo freezing and is a potentially dangerous source of external ice crystals (R. T. Cook, 2004; Storey, Storey & Churchill, 2007; Udaka, Goto & Numata, 2008). As ectothermic animals, slugs can respond to temperature variation by using physiological (e.g. employing thermal protective proteins [Storey, Storey & Churchill, 2007]) and behavioural adaptations to regulate their internal temperatures (R. T. Cook, 2004; Storey, Storey & Churchill, 2007). One behavioural adaptation is to take shelter during unfavourable environmental conditions (drought, heat, frost, etc.) where extreme air temperatures can be largely avoided (Riddle, 1983; Wiktor, 2000). In temperate regions, the snowpack acts as an insulator, keeping temperatures in the subnivean environment close to 0°C during winter, although low temperatures of up to -8°C can occasionally occur at this level (Storey & Storey, 1996). Thus, to survive winter, slugs might either find frost-free sites or use freezing tolerance mechanisms, by, for example, producing and accumulating osmolyte molecules which help to protect cells against dehydration and stabilise cell membranes (Storey, Storey & Churchill, 2007). Furthermore, water loss due to epidermal evaporation is a physiological event that slugs cannot prevent (Rollo, 1991), and over long periods of time it could lead to desiccation (Cowie, 1985). Microhabitat selection is therefore crucial for survival (McQuad, Branch & Frost, 1979). To avoid hot soil surfaces, snails use the vertical thermal gradient in the environment by burrowing themselves into the ground or by climbing (McQuad, Branch & Frost, 1979; Dittbrenner *et al.*, 2008) and they restrict activity to favourable daytime periods (Dittbrenner *et al.*, 2008). It can be expected that slugs use similar adaptive behaviours. Additionally, slugs are reported to also use another behavioural strategy called “huddling”, in which they form dense aggregations of several conspecifics (A. Cook, 1981). Richter (1976) suggested this could have a social thermoregulatory function, as by huddling slugs reduce the surface area exposed to air (A. Cook, 1981) thereby reducing the evaporative water loss which is associated with cold temperature (R. T. Cook, 2004) or dry conditions (A. Cook, 1981; Prior *et al.*, 1983).

The Kerry slug *Geomalacus maculosus* Allman 1843 is one of four species in the genus *Geomalacus* of the Family Arionidae. This species has a Lusitanian distribution and is only found in western Ireland and in north-western Spain and Portugal (Mc Donnell *et al.*, 2013). It is protected by Irish national conservation laws under the Wildlife

Amendment Act 2000, as well as under the EU Habitats Directive 92/42/CEE and the Bern Convention (ETS No.104 Appendix II). In Ireland, *G. maculosus* is regarded as crepuscular, although some observations have reported the animal active in daylight during and shortly after rain, while in Iberia the species is considered entirely nocturnal, possibly due to the higher temperatures (Platts & Speight, 1988). Very little is known about the tolerance of this species to different temperatures, but Patrão *et al*. (2015) suggested that "maximum temperature of the warmest month" was one of the most important variables explaining the distribution of the species, and that in humid conditions *G. maculosus* could survive temperatures up to 29°C. While Van Helsdingen *et al*. (1996) reported that the species in Ireland was mostly active during the winter and aestivated for part of the summer, other, more recent studies have captured slugs throughout the summer within Irish conifer plantations (Johnston *et al.*, 2017, 2018; Reich *et al.*, 2017). Platts & Speight (1988) observed active young specimens even after snowfall and at temperatures rarely above freezing. Thus, it is evident that this species possesses some mechanism that enables it to survive a wide range of temperatures. The ecophysiological factors that limit the distribution of *G. maculosus* are not fully understood (Van Helsdingen, Willemse & Speight, 1996; Reich *et al.*, 2015) but they are essential for its conservation management. It is also extremely important to assess the potential impact that changes in temperature could have on such vulnerable species particularly in the context of global warming. Thus, the aim of this study is to fill existing research gaps by examining the tolerance of the slug to extreme heat and cold.

Adult specimens of *G. maculosus* were collected under licence in conifer plantations in the west of Ireland (Cos Galway and Kerry). Slugs were collected between October and November 2015 and again in April 2016. Slugs were kept at an ambient room temperature of 20° ± 5°C in plastic boxes (16 cm x 11 cm x 5 cm; five slugs per box) with moistened tissue paper and were given carrot as food. Prior to experiments (conducted in April and May 2016), they were kept for at least one week in water baths set at a constant temperature of 15°C to facilitate acclimatisation. This was done following Roy (1963) who suggested that five days are sufficient to complete the metabolic thermal acclimatisation in *Arion circumscriptus* slugs exposed to different temperatures. For the experiment, a mixture of both phenotypes (black-brown and yellow-brown) of 120 adults which were about equal in size was used. Slugs were divided into eight groups of 15 individuals and each group was subjected to a different temperature (40°C, 38°C, 36.5°C, 35°C, -3°C, -6°C and -9°C) for one hour. The control group was kept at 15°C as this temperature is thought to be optimal for this species (I. R. *pers. observ.*). Within each group, slugs were placed in five Petri dishes (9 cm diameter) containing three individuals each, following the Getz (1959) protocol so that slugs could engage in huddling. Petri dishes were exposed one at a time to the desired temperatures. Several replicates were conducted per day, using different Petri dishes and different treatments in a randomized order. Three layers of filter paper (Whatman No. 1) were placed on the base of each Petri dish. For heat tolerance experiments, the filter papers had previously been moistened with 5ml of tap water to guarantee an adequate humid environment and to reduce stress due to evaporative cooling mechanisms (Udaka, Goto & Numata, 2008). However, water was not added for the cold tolerance experiments to avoid inoculative freezing due to potential contact with frozen water. In all experiments, the Petri dishes were sealed using a thick layer of Vaseline® around the borders and Parafilm® to maintain constant levels of humidity inside the Petri dishes. Once the desired temperature was reached, the Petri dishes were placed in an incubator (LMS 410XAL) (for temperatures between 15°C and 40°C) or in a freezer (Zanussi Freezone ZFC61/27) (for temperatures between -3°C and -9°C). A Taylor maximum-minimum thermometer was used to ensure that stable temperatures were maintained throughout the experimental period. After the exposure, the response of slugs to tactile stimulation was examined, i.e. a positive response was recorded by movement of the foot after touching it with a wooden cocktail stick (6.5cm long, Mobi Lock©) (Dittbrenner *et al.*, 2008). Subsequently, slugs were transferred to clean containers which were placed in water baths maintained at 15°C (control temperature) and exposed to natural photoperiods (average 15 hours light:9 hours dark). Mortality was then recorded after 24 hours and again after seven days following the experiment. The criterion for survival was taken as either a response to tactile stimulation (McQuad, Branch & Frost, 1979; Riddle, 1983; Dittbrenner *et al.*, 2008) or as the movement of mantle muscles around the breathing pore. Statistical inferences were performed using MINITAB 17 (2010). Differences in the survival of the slugs were assessed using a one-sided Fisher's exact test, which is based on hypergeometric distribution calculations.

The mortality rates plotted against exposure temperatures are presented in Figure 1, indicating the range of temperatures that *G. maculosus* can withstand for one hour without any effects on its mortality. Individuals of *G. maculosus* which were exposed to 15°C, 35°C and 36.5°C did not show any stress symptoms such as dehydration (i.e. wrinkled body surface) or overproduction of mucus (O'Hanlon, Williams & Gormally, 2019). Furthermore, they all responded positively to tactile stimulation, both immediately and 24 hours after treatment. Although after seven days some mortalities were recorded within all groups, mortality rates (after 24 hours and seven days) were not significantly higher in each of the two groups exposed to 35°C and 36.5°C compared to the control (15°C after 24 hours and seven days respectively; Table 1). In contrast, mortality rates both after 24 hours and seven days following exposure were significantly higher in groups exposed to 38°C and 40°C compared to the respective controls ($P<0.001$, Table 1). Furthermore, slugs that recovered from these treatments showed severe symptoms of stress (as described above). Dead individuals were found lying on their sides and fully extended with a wrinkled surface, indicating dehydration (Figure 2, Supplementary Data). Eight individuals which, under the criterion of movement of mantle muscles were classified as "alive" 24 hours after the treatment at 38°C, did not respond to tactile stimulation and died three days after the treatment exposure. Thus, we show that *G. maculosus* can tolerate heat of up to 36.5°C without suffering from any acute damage. Nevertheless, the significantly higher mortality recorded for the group exposed to 38°C shows how a small increase in temperature (1.5°C) can have serious consequences for this

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species. Getz (1959) recorded a maximum heat tolerance of 35°C for *A. circumscriptus* and 36°C for *Deroceras laeve* and *Deroceras reticulatum*, three species that are widespread across Europe and North America. It has to be taken into account that in the Getz (1959) study results were recorded after an exposure of only 15 minutes, while in our study *G. maculosus* was exposed for one hour. *Geomalacus maculosus* also demonstrates a better ability to withstand higher temperatures in comparison to *Agriolimax agrestis* which does not tolerate the exposure to 35°C for one hour (Carrick, 1942) and to another globally widespread species, *Lehmannia valentiana*, juveniles of which were unable to survive exposure to 36°C for one hour (Udaka *et al.*, 2007).

Geomalacus maculosus presented an appreciable degree of resilience to freezing, as the mortality of the groups exposed to -3°C and -6°C, both after 24 hours and seven days following exposure, was not significantly higher than the mortality of the corresponding control groups. In contrast, the mortality rates both 24 hours and seven days following exposure to -9°C were significantly higher than the mortality rates of the respective control groups ($P<0.005$, Table 1). Immediately after exposure to below-freezing temperatures, all slugs showed severe stress symptoms: most individuals were covered in a thick layer of yellow frozen mucus and presented some degree of frozen tissue, which made it difficult for them to move (Figure 3, Supplementary Data). Individuals that died after exposure to freezing temperatures presented a contracted body with no distinct shape. These symptoms were milder at -3°C, where slugs had only small amounts of frozen tissue and were still able to crawl and to respond to tactile stimulation. Slugs exposed to the two lowest temperatures presented a fully hardened body with retracted tentacles, and only two slugs at -6°C and one slug at -9°C still responded to tactile stimulation immediately after the experiments concluded. However, after 24 hours, eleven and nine slugs respectively revived upon being thawed, the majority responding to tactile stimulation and not displaying stress symptoms. Although two individuals of *A. agrestis* were described to survive the exposure of one hour to -5°C (Carrick, 1942), other species had a lower resistance to freezing temperatures: individuals of *D. reticulatum* and *A. circumscriptus* died when exposed for one hour to temperatures of -1.5°C and -1.2°C respectively and spring individuals of *D. laeve* showed a 100% mortality when exposed for one hour to -4°C (Storey, Storey & Churchill, 2007). The authors also described similar stress response to cold temperatures as the one reported here: a shapeless body covered by a copious amount of thick yellow mucus. Hargens and Shabica (1973) reported an intertidal limpet species secreting profuse mucus that encapsulated the animal when submitted to progressive freezing and concluded that it could have a protective effect, inhibiting the growth of ice crystals. It can be hypothesized that the thick layer of mucus in slugs has a similar function, acting as isolating material. Moreover, while excreting a copious amount of mucus could be a mechanism to expel internal water to prevent cell damage (Storey & Storey, 1996), a more recent study by Udaka, Goto & Numata (2008) implies that, given the susceptibility of slugs to inoculative freezing, frozen mucus in contact with their skin could pose a serious threat. Clearly, further studies on the function of the thick mucus layer secreted by certain molluscs when exposed to freezing temperatures are required. Although all *G. maculosus* specimens were frozen solidly after being exposed to -6°C and -9°C, 73.33% and 60% respectively revived after thawing. This agrees with the observations of Storey *et al*. (2007) on *D. reticulatum*, *A. circumscriptus* and *D. laeve*, of Getz (1959) on *D. laeve*, and of Carrick (1942) on *A. agrestis*. In a habitat where frequent freeze-thaw cycles occur, as is the case of some of the habitats of *G. maculosus,* it may be advantageous to be able to supercool or to tolerate some freezing (Block, 1982). This agrees with our findings and supports the idea of Ansart *et al*. (2002) in which ice formation could occur in some parts of the organism's body without threatening its survival. Thus, we hypothesize that in the field, during cold periods, *G. maculosus* could withstand some ice formation, recovering later when the temperatures are milder. This is supported by reports of several individuals of this species in the field surrounded by snow and ice (Platts and Speight 1988).

Although the results presented in this study suggest that *G. maculosus* might display a better tolerance to extreme temperatures than other slug species tested, it is worth noting that care should be taken when comparing different studies, as differences in the experimental setups might have influenced the reported tolerance. In some of the above-mentioned studies, slugs were exposed individually to the tested temperatures. This could have prevented the individuals from huddling, a behaviour that might occur when exposed to adverse conditions, and that might enhance their ability to withstand extreme temperatures, although further investigation of huddling behaviour is required to clarify this. In our study, the humidity within the Petri dishes that were subjected to hot temperatures was deliberately set higher than in those subjected to the low temperatures. Survival chances of the slugs would likely be diminished if this was reversed due to increased evaporation under hot and dry conditions and inoculative freezing under humid and cold conditions. Furthermore, due to the above-mentioned differences a direct comparison between the cold and hot treatments in this study is not possible. When considering tolerance to extreme temperatures, it is important to take into account that different variables such as exposure time, photoperiod, physical conditions, age of the individuals, and interrelations of stress factors (e.g. humidity variation with temperature) might influence the survivorship (Getz, 1959; McQuad, Branch & Frost, 1979; Riddle, 1983; Ansart, Vernon & Daguzan, 2001). Therefore, it is difficult to experimentally determine the exact conditions in which the animal might survive in the field (Getz, 1959). Furthermore, to determine fitness repercussions, essential functions such as growth and reproduction should also be assessed in the long term following exposure (Riddle, 1983; Ansart, Vernon & Daguzan, 2002). Nevertheless, this preliminary study shows, for the first time, the temperature tolerance limits of the EU-protected slug *G. maculosus*. The data provide the baseline parameters for future investigations which should take some of the above factors into account to gain a more comprehensive picture on the long-term tolerance to extreme temperatures. Our results also inform future studies modelling the suitability of habitats or areas for *G. maculosus* in the light of current and future climate change.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Figure 1. Mortality rates of *G. maculosus* exposed to different extremes temperatures (for each group n=15). Note that mortality rates on day seven were corrected using Abbott's formula.

Supplementary Data

Figure 2: Three individuals (a, b, and c) of *G. maculosus* recovered after the exposure at 38°C for one hour. A conspicuous amount of yellow mucus (indicated by the black arrows) can be seen on the filter paper. Slugs were found in the typical position for heat exposure: lying on their side, with the body elongated. The surface body of individual (a) presents wrinkles.

Figure 3: Two individuals of *G. maculosus* recovered after the exposure at -3°C for one hour. The arrow indicates frozen mucus and tissue on the slug's tail.

Table 1. Mortality rates of the groups exposed to different temperatures at 24 hours and seven days after exposure. All P-values (* P <0.05, ** P<0.005, *** P<0.001) are the result of the comparison between each treatment (mortality after 24 hours and seven days) and the control (mortality after 24 hours and seven days respectively) using Fisher's exact test; n=15 for each treatment. As there was control mortality seven days after the exposure, the mortality rate at this time point was corrected using Abbott's formula.

Note that as all the individuals of the group exposed to 40°C were found dead 24 hours after the exposure, it was not possible to calculate a change in the mortality rate at the seventh day.

	24 hours after the exposure			Seven days after the exposure			
Temperature	Number of deaths	Mortality rate	P-value	Number of deaths	Raw mortality rate	Corrected mortality rate	P- value
40°C	15/15	100%	<0.001***	15/15	n/a	0%	<0.001***
38°C	7/15	46.67%	0.003**	15/15	100%	100%	<0.001***
36.5°C	0/15	0%	1	3/15	20%	0%	0.674
35°C	0/15	0%	1	1/15	6.67%	0%	0.299
15°C (control)	0/15	0%	-	3/15	20%	0%	-
-3°C	0/15	0%	1	3/15	20%	0%	0.674
-6°C	4/15	26.67%	0.05	8/15	53.33%	41.67%	0.064
-9°C	6/15	40%	0.008*	9/15	60%	50%	0.003**