Husbandry guidelines for the safe brumation of two lacertid lizard species (*Iberolacerta monticola* and *Podarcis lusitanicus*) in laboratory conditions

Frederico M. Barroso[a,b,c] *

[a] *CIBIO, Research Centre in Biodiversity and Genetic Resources, InBIO, Universidade do Porto, Campus Agrário de Vairão, Rua Padre Armando Quintas, Vairão 4485-661, Vila do Conde, Portugal*

[b] *Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, R. Campo Alegre, s/n, 4169-007, Porto, Portugal*

[c] *BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal*

*Corresponding Author: frederico.m.barroso@gmail.com

Abstract:

Lacertid lizards are often used for a range of laboratory studies in reptile behaviour, physiology, ecology etc. However, there is a distinct lack of primary information regarding the husbandry of these animals under captive, laboratory conditions. This lack of information is even more apparent when looking for standard husbandry best practices for the brumation of small lizards in a captive setting. Hence, when faced with the need to overwinter captive populations of two lacertid lizard species from a montane environment (*Iberolacerta monticola* and *Podarcis lusitanicus*), the need arose to establish a conservative protocol that would allow the animals to safely go through their seasonal activity cycle (and brumate) in a controlled laboratory environment. This protocol therefore addresses that gap as it aimed to simulate a simplification of the conditions thought to be experienced by these animals while brumating. Ultimately, through this protocol, these species were successfully overwintered in a laboratory setting without any casualties (during the brumation period) and with no significant effect on the body condition of the animals. Hence, this protocol hopes to provide a baseline framework, on which other studies may capitalise, build upon or adjust, for the safe brumation of lizards in a laboratory setting.

Background:

Many animals resort to some level of dormancy in order to cope with periods of scarce resources or harsh environments. Hibernation in mammals is a textbook example of such a period of inactivity and physiological latency. Nonetheless, analogous inactive states exist in many other groups including reptiles, where they are termed aestivation (over Summer, often observed in xeric or tropical species) or brumation (over Winter, often observed in alpine or temperate species).

Nonetheless, despite this general knowledge that these animals brumate, there is a remarkable absence of published primary information regarding the conditions in which these animals do so, either in a wild or captive setting. Furthermore, many such species, including some lacertid lizards, are often found in private or zoological colections, or commonly studied species in a captive/laboratory setting. Hence it is crucial to have best practice guidelines on the husbandry of such species, both for their active and inactive periods. This protocol wishes to address that knowledge gap by resorting to some information compiled (for other species) from private lacertid keeper forums, as well as on an informed attempt to simulate the most realistic recreation of what were the winter-time environments in the animals' native ranges, in order to suggest a replicable and adjustable methodology for the safe brumation of lizards in a laboratory setting.

Protocol aim:

The following protocol was devised in order to brumate a large number of wild lizards in a captive setting. These animals had been kept in the lab from the Autumn of 2020, when they underwent a range of behavioural and physiological tests. Given the time of the year when these finished, and the unfavourable environmental conditions for a harsh release back into the sites of capture, the animals had to be kept in the lab until the Spring of 2021. Hence, and in order not disrupt to the animals' natural cycles, it was decided to attempt to provide conditions conducive of brumation.

Ultimately this protocol hopes to provide a baseline framework for the safe brumation of lizards in a captive state. Nonetheless, caution is advised when extrapolating this protocol towards species beyond those described here and which may have different activity/physiological cycles or be exposed to different wintertime environmental conditions. Hence, it is advised that this protocol is adjusted according to the specific requirements of each species and conditions encountered by these in their natural habitat.

Furthermore, it is important to note that this protocol was devised under a lack of official husbandry/brumation guidelines for the species of interest and from the country where the study was conducted. Nonetheless, prior to deploying this protocol to any other location and/or species, a thorough search for the local official guidelines and legislation applicable to the species in question must be performed.

Animal subjects used for this protocol:

The animals in question were adults of the species *Iberolacerta monticola* (12 males, 8 females) and *Podarcis lusitanicus* (12 males, 17 females; formerly *P. guadarramae lusitanicus*) collected from Serra da Estrela (district of Guarda, Portugal). This is a montane environment (at 2000m and 1400m of altitude for each species respectively) where, in winter, there is a permanent snow cover under which the animals brumate in burrows/crevices.

Before Brumation:

-Enclosures:

Males were kept individually in separate terraria (30Lx20Wx17H cm) and provided with a small brick for shelter and thermoregulation. Females were kept in communal enclosures (46.5Lx37.5Wx23.5H cm, one for each species) with several stacked bricks to provide shelters and thermoregulation platforms.

-Food and water:

Animals were provided with water *ad libitum* in water dishes (individually for males and communal for females) and lightly misted daily to maintain environmental humidity. Lizards were fed *Tenebrio molitor* larvae, dusted with a multivitamin powder, 3 times a week. At times, crickets (*Acheta domesticus*) were made available instead.

-Photoperiod:

Natural photoperiod was provided via a diffuse glass window and an array of 150w infrared basking lights, at a 2-3m distance from the terraria, as a radiant heat source for basking. Photoperiod followed the progression that the animals would experience in the wild since the lab was at a similar latitude to the animals' sites of capture. As Winter approached, the animals became increasingly less active and less interested in the food supplied. Perhaps triggered by the naturally decreasing photoperiod, temperature or other unknown intrinsic factors. To match this, within 1 month prior to moving the animals into the brumation chambers, the time of basking lights available was gradually decreased until a minimum period of 5 hours (always concentrated around mid-day).

-Air Temperature Control:

Air temperature was maintained around 18ºC via an air conditioning (AC) unit.

During the pre-brumation period, air temperatures were also allowed to fluctuate more naturally along the day by turning off the AC from late afternoon until the following morning. As days got progressively cooler outside, so did the lab itself. Although, still providing an increasingly short window of sufficient warmth for the animals to feed and digest (through a combination of AC and basking lights). As the animals' physiology changed and their feeding drive decreased, the frequency of feeding was also decreased, to a point when animals were not fed at all (for a minimum of 1 week prior to being transferred into brumation chambers). This was to ensure that food was not consumed and left undigested during the long brumation period. Otherwise, undigested food could potentially lead to severe constipation (formation of faecoliths) or decompose causing internal infections, sepsis and, ultimately, death of the animal.

-Monitoring:

Body condition of the animals was monitored through monthly weightings to ensure the animals started brumation with an adequate condition (i.e. were neither too thin nor in a trend of sharply decreasing weight). To avoid disturbing the animals during the brumation process, during such time, animals were only visually inspected to assess their condition. Regular (weekly) weightings were only resumed post-brumation, to assess the effects of brumation on body condition and track the subsequent recovery

Brumation Set-Up:

Animals of these species are often found in the wild aggregating in the same crevices and/or basking spots. Hence, due to space constraints, animals were placed in communal brumation terraria.
It is important to note that all animals came from the same population (of each species) and that, when placed in brumation, no animal was showing signs of poor body condition or disease/parasitization. When this is the case, it is advisable to either quarantine the animal(s) or not proceeding to brumation should the animal be too weak.

-The Terraria:

Each brumation terrarium housed 8-12 animals of the same sex and species. Additionally, only animals from the same population should be housed together, and any animal showing signs of bad body condition or disease/being parasitized must be kept isolated or even considered to exclude from brumation.
The terraria initially used were simple, transparent plastic storage boxes (40Lx29Wx15H cm, 12 litres) with a locking mechanism for the lid. Presence of a locking lid is essential to prevent animals from escaping as they may be strong enough to push their way out if not properly locked. The lid itself was modified by creating many evenly spaced ventilation holes across its entire surface (diameter ≤ 5mm but adjust to species size; Figure 1). Taller boxes (40Lx29Wx25H cm, 20 litres) were subsequently used in a further study (in progress) on the same species, which enable more volume and the ability to create more shelters and a vertical gradient of temperature and humidity (Figure 1).
Furthermore, in order to prevent extensive direct contact between the bottom of the terraria and the metal floor of the brumation chambers, the external bottom sides of the brumation terraria were insulated with sheets of cork (Figure 2). This is due to the fact that metal (from the chambers' floor) tends to draw away heat very fast from a body and this could increase the risk of inducing hypothermia or even killing the animals by a fast cold shock. As an additional preventive measure, wood boards were also used to lift the terraria from the metal surface (Figure 3).

Figure 1. Brumation terrarium set-up

-The Substrate:

In an attempt to create a substrate humidity gradient, each terrarium was split in half. One side took a thin (~1cm) layer of sand mixed with vermiculite (<10% volume), and the other a 1-2 cm deep layer of "bioactive substrate" (see "Bioactive Substrate" section for details) (Figure 2). A subsequent deployment of this protocol successfully recreated a more stable hydric environment by using a deeper layer of bioactive substrate (5-10cm). This provided further microhabitats for the animals to dig and brumate into, as well as better moisture retention properties (i.e. no need to spray as often, meaning less disturbance).

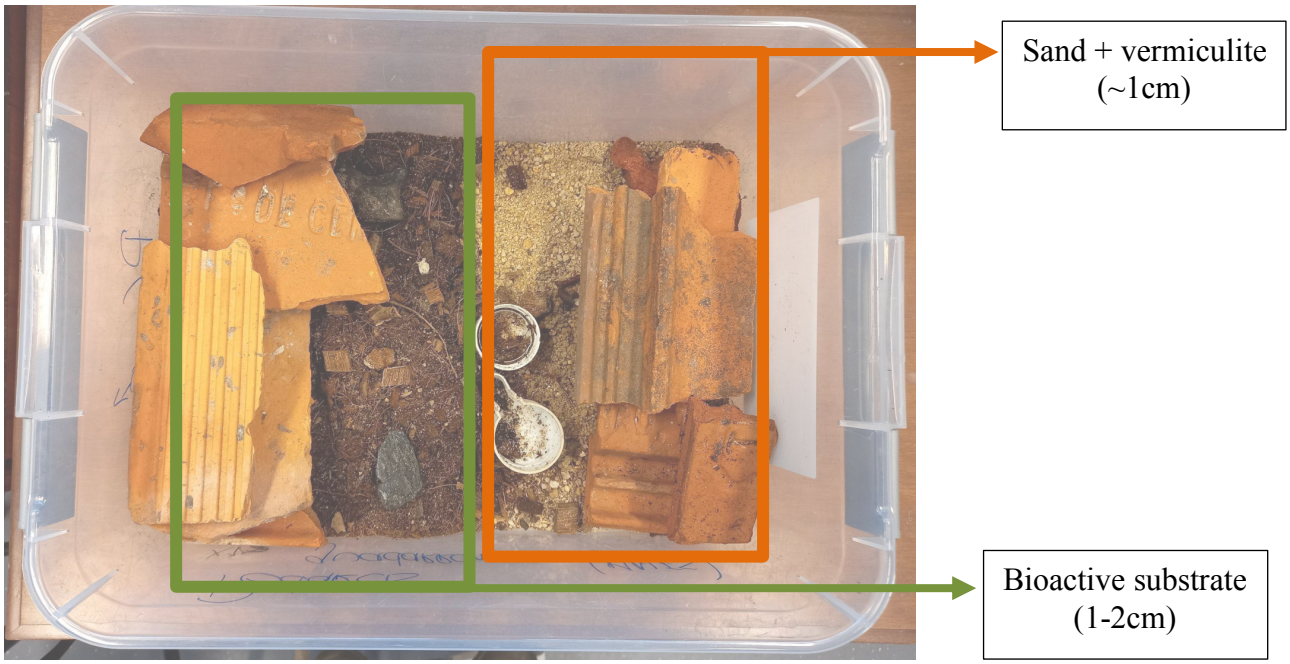

Figure 2. Substrate distribution in the terrarium.

-The Refugia:

To create refugia, layers of pieces of bricks/tiles were carefully stacked in opposite ends of the terrarium (Figure 1), leaving 1-2cm tall (species dependent) crevices between layers. Much care was taken to ensure stability of the final structure as these animals are deceptively powerful at moving even larger pieces of brick. In order to facilitate access to the animals these pieces were not glued together, and it was really strived for mechanical stability when arranging the pieces. However, gluing the pieces together is indeed the safest option to avoid any animal being inadvertently crushed. To do so, the best options would be to use 100%/Aquarium-grade silicone, "super-glue" (cyanoacrylate glue) or careful use of expanding polyurethane foam (with no additives). These however, each have very specific curing, but all eventually render completely safe and non-toxic solutions when fully cured, as is evident by their ubiquitous use amongst aquarium and terrarium keepers.

The stacks of bricks were created as tall as possible but also allowing for enough space for the animals to move between layers of bricks and between the top layer of the structure and the lid of the container. The latter was in fact very often used (particularly by the *Iberolacerta monticola).* Additionally, some free space of just soil and sand was left open, in between the stacks (Figure 1).

-Water:

A couple of water dishes were supplied in the middle of the terraria (Figure 1). These allowed the animals to drink or soak but also contributed towards maintaining a high relative humidity inside the terraria, aided by periodic misting.

-Biosecurity Measures:

All bricks and water dishes were previously sterilised by soaking and washing them in solution containing bleach (household cleaning bleach ~5-10mL in 10L of water). These were then thoroughly rinsed and the water (an any remaining bleach) left to evaporate over the course of a couple of days.

The bricks were then further sterilised by placing them in an oven at 80ºC for a minimum of 30 minutes. The substrate systems also underwent a heat-sterilisation process (baked at 80ºC for over 1 hour). However, after such treatment, the bioactive substrate must once again be rehydrated and recolonised with native isopods and collembola (the bioactive clean-up crew; see "Bioactive Substrate" below).

-The Brumation Chambers:

With regards to the brumation chambers themselves, the ideal would be to use climatic/environmental chambers where the user has full control over the set environmental conditions (temperature, humidity, light intensity and period, etc.). However, these are prohibitively expensive and hence cheaper alternatives were devised for this protocol.

The initial approach, here described, resorted to the used of laboratory incubation chambers (Binder KB53, Binder Inc., USA; temperature range: -5 – 100ºC, capacity: 51L) (Figure 3 right). The limited space available in each incubator meant two of such units had to be used to house all the terraria. However, larger models do exist and would be recommended.

The incubators were set to a fan speed ≤30 (on a scale of 0 to 100) and their high precision of control of the conditions meant that no significant difference would be expected between the environment experienced inside both incubators. These have the further advantage of allowing for automatic cycles of varying temperatures to be programmed, which could be useful under certain scenarios. Nonetheless, for the purpose of this proof-of-concept, temperatures were manually set as required.

The inner, glass door of the chambers was closed but the external (metal) door was always left open to allow some photoperiod for the animals. Additionally, an array of 70W incandescent lights was placed facing the incubators from ~1.5 metre distance (Figure 3 left) and programmed to be on for 5 hours in the late morning to mid-afternoon. These reinforced the photoperiod (provided by the lab's window) but also provided a short window of basking opportunities for the animals, should they choose to take advantage of it.

Any holes larger than 1cm in diameter leading to the internal components of de incubators (e.g. access to the fan) were covered with fine netting to account for the eventuality of an animal escaping into the fan/internal compartments of the equipment.

Figure 3. Array of lamps and lab window that supplied the photoperiod and basking opportunities for animals during brumation (left); Binder KB53 incubators used as brumation chambers (right)

Alternatively, made use of a commercial refrigerator for beverages/cakes (Figure 4 left). Unlike household refrigerators, these tend to have a transparent glass door which proves crucial for manipulating the photoperiod as well as for visual inspection of the terraria without the need to open the unit (which would affect its temperature). These are an even cheaper option than laboratory-grade incubators and allow for a sufficient temperature control usually around the 0-10ºC range (sufficient for the species in question but may not be adequate for all species).

However, this control is neither as precise nor as accurate as the incubators, hence it is important not only to measure the temperatures before adding the lizards but also to monitor it through time. Furthermore, the compressor in these refrigerators does not run continuously and usually has a threshold of around +/-2ºC (depending on the model) around the temperature it's set to, around which it fluctuates in a cyclic manner

(see graph in Figure 4). Hence it is very important to measure this in order to select the best setting before use, but also to closely monitor these fluctuations during the use (e.g. via temperature dataloggers).
Additionally, these refrigerators do not usually allow control of the air flow. They will have an air flow vent from which cold air is blown into the unit to cool it down. This vent is usually found in the top of the unit, as cold air is then expected to sink. However, this means that a vertical gradient of temperatures could arise inside the unit. To avoid this, simple, quiet USB fans, easily be acquired online (Figure 4 left), were run continuously at the bottom of the unit promoting a more homogenous distribution of temperature. Nonetheless, close monitoring of temperatures along the different levels of the unit is highly recommended before and during its use.
Ultimately, even though no information was available on the exact temperature and their stability inside the natural hibernacula of these species, these fluctuating and less precise conditions where successfully used by Sreelatha and Barroso *et al.* (manuscript in progress), for the same two species, in the follow-up study aforementioned. The refrigerator used for such study was the model "Magnus M&S D 372 SCM 4C" (temperature range: 0-10ºC, power: 0.35kW, Capacity: 325L) (Figure 4 left).

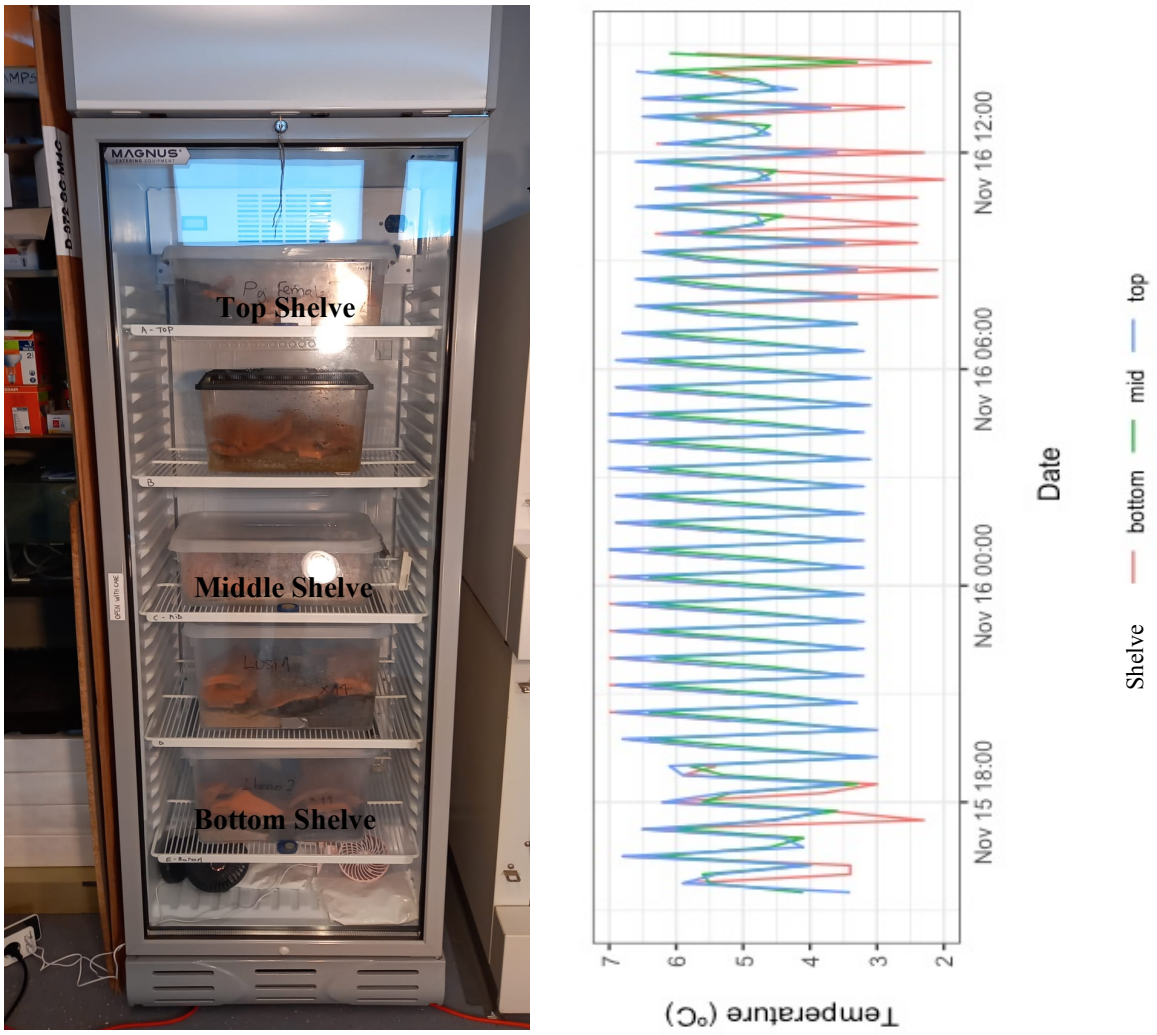

Figure 4. Commercial refrigerator (left) used where fans for air movement are visible (below bottom shelve) as are the small dataloggers (small grey circle inside blue cap in top, middle and bottom shelves) used to monitor temperature profile; Temperature profile (left) of each shelve measured showing temperature fluctuations over a 1-day period.

During Brumation:

-The Conditions:

After being placed in the brumation chambers, the temperature was gradually decreased from 15ºC (ambient temperature) to 5ºC over a period of 2-4 weeks. 5ºC temperature and fan speed = 30 were the conditions left for the remainder of the brumation period. Natural photoperiod was maintained by indirect sunlight from a frosted glass window and supplemented by an array of 70W incandescent lights (Figure 3 left) facing the incubators from 1.5 metre distance. The latter were programmed to be on for 5 hours in the late morning to mid-afternoon. Additionally, feeding was entirely suspended throughout the brumation period.

Throughout the entire brumation period, the incubator glass door was periodically opened and allowed to ventilate (to exchange the air inside) for a couple of minutes (3 times a week when using the [smaller volume] incubators, once a week for the larger refrigerator). At this point the lab's AC was turned off so outside temperatures did not represent such a massive difference to that of the incubators. Furthermore, the incubators rapidly recovered the desired temperature once the door was closed.

-Regular Monitoring:

Once a week, all the terraria were opened and visually inspected (without disturbing the animals, unless necessary). Several aspects were checked:

- Soil moisture – the bioactive soil should be moist (not wet, and still loose; Figure 8). If such was not the case, then the terrarium was lightly misted. In fact, most of the times the terraria were actually too wet (from condensation). This was later addressed by increasing the number of holes in the lid of the terraria as well as adding some (very few and very small <2mm diameter) holes on the sides of the terraria. Small holes in the bottom of the terraria should also be considered in order to drain excess water that may accumulate under the substrate system.

- Presence of ice formation (in the incubator/refrigerator or in the terraria) – the brumation chambers and the terraria were visually inspected for indications of ice. This should be avoided at all costs to prevent animals from freezing or suffering frostbite, as well as to avoid damage to the unit.

- The water dishes still have water and its clean (refilled/ replaced as needed with tap water).

- If there are signs of bricks being dislodged and if so double check that no animal is trapped (which never happened) and re-secure the bricks in question.
  In the animals that are visible (i.e. avoid unnecessary disturbance to the terraria), check for general health signs such as apparent loss of mass, discharge from mouth or nose, presence of fungi/external parasites/wounds/frostbite, etc. This must be done with minimal disturbance to the animals (i.e. without touching them unless a problem was indeed suspected). Nevertheless, issues with the animals during the brumation period were rarely seen. However, had there been any such suspicion, the animal(s) in question would have been removed and placed in an individual terrarium to avoid propagating the condition. Depending on the severity of the situation, terminating brumation should

be considered to allow the animal to attain body temperatures high enough to resume feeding and hence speed the recovery/healing process. This, however, should be avoided as suddenly terminating the brumation may present a big physiological shock to the animal.
This visual inspection was easier for *Iberolacerta monticola* since they tended to lodge themselves between the top bricks and the lid of the terraria (Figure 5), as opposed to the *Podarcis lusitanicus* which more often hid in between the layers of bricks.

- Temperature and humidity measurements from data loggers (some inside the brumation chambers but out of the terraria, others inside the terraria themselves) were downloaded and checked for any abnormalities or concerns.

Figure 5. *I. monticola* (males) in their brumation terrarium during one of the periodic checks. They were often found clumped together at the top of the terraria.

-Managing Relative Humidity:

It was also not infrequent to observe large amounts of condensation happening both inside and outside of the incubators/refrigerator doors. This did not necessarily represent an issue to the brumating animals, yet it often led to the pooling of some water either inside the chambers or on the floor of the lab, potentially leading to water damage to those. This could result from maintain the brumation terraria too humid, at which point the amount of misting must be addressed.
Alternatively, it could also result from humidity in the outside air condensing in the cold surfaces of the incubators/refrigerators. To address these, it was opted to use a dehumidifier in the laboratory and an air circulation fan pointing towards the door of the chambers. These promoted the evaporation of any water condensing on these surfaces.
Issues with condensation inside the units were dealt by adjusting the amount of misting as well as by lodging a small piece of cardboard in the door to leave a small crack open and promote some air exchange with the outside conditions (managed by the dehumidifier and the fan) which must be kept as cold as allowed by the AC unit. This approach, however, must be monitored closely as it may promote unstable or undesirable temperature conditions inside the units.

-Re-establishing Activity:

Towards the end of the desired brumation period, the reverse protocol was employed to "wake up" the animals. Temperature was gradually raised from 5ºC to 15ºC inside the chambers. Additionally, while animals remained under natural photoperiod, the amount of time that the basking lights were on was gradually increased. After 2-4 weeks of this gradual increase, the animals were returned to individual (males) or communal (females) terraria with access to bricks for refugia, water dish, 150W IR basking and UVB lights (Figure 6). Again, the basking period was also gradually returned to the 8-10hour period supplied before brumation, as was the food supply (gradually increased from once a week to once every other day). The latter was done based on the animals' response when feeding. If the animals were seen to be active and readily taking food, then the regularity of feeding was increased. Animals were fed with multi-vitamin dusted crickets (*Acheta domesticus,* preferred option) or mealworms (*Tenebrio molitor*). Standing water was provided *ad libitum* by a water dish in each terrarium, and the terraria misted daily/ every other day.

Figure 6. Set-up of the individual and communal terraria used to house the animals before and after brumation. Array of infrared heat/ basking lamps also visible.

After Brumation:

Animals were kept in the incubation chambers from the beginning of December 2020 to the end of March 2021. This is very likely a shorter period than that what they would usually brumate in the wild. This was due to the fact that these animals were still undergoing some tests at the time they would typically brumate and then they still had to be slowly acclimated into brumation conditions. Furthermore, the decision was taken to "wake them up" from brumation earlier than what would naturally occur for several reasons namely the fact that this was the first time these conditions were devised, and it was thus far unknown if the conditions were right and if the animals were in fact losing condition or not, beyond their encouraging physical appearance. Additionally, it was also the intention that, as soon as conditions in the wild were favourable, animals could immediately be returned to their site of capture in the best body condition possible. Hence a few weeks were taken to ensure the animals were well fed, properly active and in good condition to be released.

-Post-Brumation Body Condition and Mortality:

All animals survived the brumation period and emerged in good body condition with no noticeable change in the body condition before vs after brumation (Figure 7). However, a few (3-5) weeks after the end of brumation some unexpected mortality was observed. 1 male *Iberolacerta monticola* died of unknown causes as well as some (6 from both species in total) of the females died from apparently a multitude of causes. A couple seemed to develop some fungal growths while others were simply found buried in the substrate of the communal terraria or under the tiles just dead (no signs of being trapped).

Given that no necropsy or pathological analyses were performed on the dead animals, precise cause of death is now difficult to pinpoint for these animals. The conditions (housing, food, environmental) in which the animals were housed post brumation matched those in which they had lived for circa 2 months prior to brumating, with no registered incidents of disease or death.

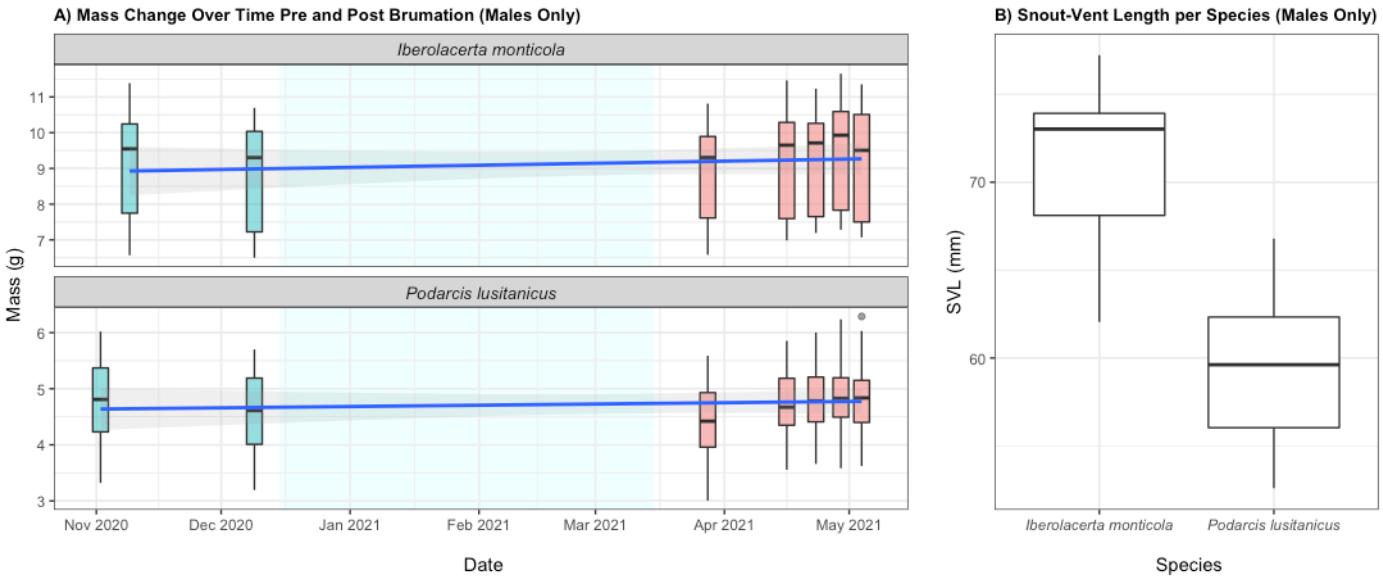

Figure 7. A) Mass change over time for males of both species showing no difference between pre (blue) and post (red) brumation mass. Light blue shaded area corresponds to the brumation period;
B) Snout-to-Vent lengths of males of both species showing the difference in size between the species.

Furthermore, the animals appeared in good condition (visual inspection for females, visual inspection and tracking of the weight of the males) and were checked at least once a week. On all such cases, death was quite sudden and unexpected. Nonetheless, a study by Alves de Matos et al (2013; https://doi.org/10.1017/S1431927613001773) has shown (in these and other Iberian lacertid species) and increased incidence of iridovirus-like viruses in post-brumating lizards, affecting their survivability. Although this was not monitored during the development of this protocol, it could explain the sudden susceptibility (and death) of the animals post-brumation and further reiterates de importance of a good sanitary protocols.

Hence, for future applications of this protocol, it would be advisable to keep all the animals in individualised and simple (i.e. just water dish, refugium and basking platform, no soil) terraria post hibernation. This would make it easier to maintain a more sterile environment as well as to track each individual's feeding drive and health progress over the course of a few weeks (before contemplating further experiments or release), thus enabling easier containment of potential diseases that could spread across the captive population.

Bioactive Substrate:

The composition of the bioactive substrate described here is mostly based on personal experience and inspiration from the terrarium/ reptile husbandry community. This substrate system has proven functional on many occasions and for a range of applications namely as substrate for terraria as well as substrate for lizard nest boxes.

-Composition:

The (approximate) composition of the substrate is as follows (all percentages relate to volumes):

- 30% coconut husk (terrarium grade, usually comes dehydrated so water must be added prior to use)
- 30% organic potting soil (here it is important to make sure that there are no additives - pesticides, herbicides or chemical fertilisers - on the chosen soil)
- 20% sphagnum moss mulch (often found in garden centres, for orchids and other epiphytes. Again, must ensure there are no additives and may need to be rehydrated before use)
- 10% fine silica-based sand (not sea/beach sand as this would have salt and not calcareous sands – although this depends on the species being kept - either. The ideal is the washed river sand used to make cement or the one used for swimming pool filters)
- 10% crushed leaf litter/organic matter (could be collected from a garden as long as there are no pollutants or chemicals being used, must then be washed - ideally boiled for 15 minutes - and coarsely crushed/shredded. Leaves from toxic plants must also be avoided)

To create the substrate system, the components are thoroughly mixed and water is added until the soil has a moist/fresh consistency (without being wet or soggy; Figure 8). After mixing all the components, the substrate was placed in an oven at 80ºC, distributed in flat trays, for a couple hours in order to ensure that any living organism (potential parasites, invertebrates, seeds/fungi that could germinate/grow in the soil) was killed. The procedure was carefully monitored to ensure nothing burned/combusted. Naturally, this also dehydrated the substrate, hence, after cooling, the soil was rehydrated (ideally with dechlorinated water) into the above described condition.

-The "Clean-Up Crew":

It is essential to colonise the soil with a "clean-up crew" to maintain soil health, degrade organic matter and prevent fungal growth. Isopods and collembola are the staple of such crew but other invertebrates such as earthworms may be considered depending on the desired outcome. For these there are 2 options:

1) Order them from terrarium shops - both collembola and isopods are widely available as "clean-up crew" for terraria in the pet trade. The issue is that these are usually tropical species and hence require slightly higher temperatures and relative humidity in order to prosper. Furthermore, if these are to be applied to a population of animals that will later be released in the wild, it might not be ideal to expose these lizards to the non-native clean-up crew as they may inadvertently end up carrying these and introducing them into the wild;

2) Collect them from the wild (ideally from the lizards' native range) - this would be the preferred method mostly due to the fact that the lizards kept in the lab are wild animals which will at some point be returned to the site of capture. Hence, by using a native clean-up crew one reduces the risk of potentially introducing non-native species to the wild (although, the chances of the purchased clean-up crew surviving in the wilds of Europe are relatively low, given that they tend to be tropical species). To collect isopods from the wild, simply lift some decomposing wood and they should be easy to find. To collect collembola, several methods are used by terrarium keepers, most of which did not prove very efficient. In the end, my own and preferred method relies on getting some activated carbon (for aquaria) or coal (for barbecues), soaking it in drinking water for 10 minutes and then placing it inside a perforated plastic box with many small (~2mm) holes (but not on the lid, to protect it from the rain). This can then be left in a shaded and humid area of a garden (or ideally, next to rotting wood or in a compost heap) for a couple of weeks. Collembola (and other beneficial invertebrates) will naturally colonise the lumps of coal, these can then be mixed in with the substrate mix, making it bioactive.

-General Maintenance:

The soil should be kept in a container with a perforated lid that allows breathability but also maintains some humidity. It is then very important that the soil is maintained well aerated (by frequently turning it around) and at an optimum hydration level (moist, not wet/soggy). The latter should be achieved by regular spraying ideally with dechlorinated water (or tap water left to stand in the air for 24-48hr). This is important for the good health of the clean-up crew (collembola and isopoda). Some calcium should be made available for the isopods to thrive. This can be achieved by periodically adding a washed and shredded cuttlebone or reptile calcium supplement (as available in pet shops). The soil may need regular top-ups of clean-up crew, especially if left unattended for extended periods of time (e.g. if it dries out).

After use, the soil can be recycled indefinitely. However, between uses, it should always be sterilised (by baking it at 80ºC) before being re-mixed in with the clean batch (where it will be recolonised by the clean-up crew). Whenever possible, the used soil should also be cleaned of as many "foreign bodies" as possible, such as visible faeces, shed skins, etc. This is mostly to avoid any scents or chemical cues potentially affecting the future users of the substrate.

Note: In case of mite (or any parasite/fungal) outbreaks, removed and discard all the substrate from all terraria and from the stored batch. Ideally, clean everything very thoroughly and then re-start the bioactive substrate colony.

Figure 8. Photo of the bioactive substrate in its ideal hydration level.

Acknowledgements:

This protocol was developed resorting to animals captured for another study under the umbrella and supervision of Miguel A. Carretero's (from the Functional Biodiversity research group) projects, funded by *Fundação para a Ciência e a Tecnologia* (projects PTDC/BIA-CBI/28014/2017 and 2022.03391.PTDC) and led by Frederico M. Barroso and Senka Baškiera. FMB was supported by a PhD grant (SFRH/BD/147535/2019) from *Fundação para a Ciência e a Tecnologia* and SB was funded by the *Erasmus+* programme of the European Union. Animals were caught under the capture and captive maintenance permits supplied by *Instituto da Conservação da Natureza e das Florestas* (Portugal) (process reference nr: 41205/2020/DCNB/DAN; permit nr: 805-808/2020/CAPT e DET) and returned to their site of capture after the protocol was devised. I thank the many members and collaborators of the Functional Biodiversity research group (at CIBIO research centre) for vital help in capturing the animals in the field. I thank Cristiana I. Marques for all the invaluable suggestions regarding the structure of the protocol. Many thanks to Lekshmi B. Sreelatha for her comments on an earlier draft of this protocol and her contribution, along with that of Myrto Roumelioti and Gil Frias, in optimising the protocol during its subsequent use in the aforementioned follow-up study (currently in progress).