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Protocol for setting up and rearing a successful lizard room

1: Introduction

Anolis lizards are among the best-known examples of adaptive radiation and convergent evolution. As such, *Anolis* lizards represent one of the prime models for understanding evolutionary biology (Losos, 2011) and behavioral ecology (Huey *et al*., 2004). Housing and breeding experiments with anoles provide an excellent means for estimating heritability and can be a valuable resource for the study of development, adaptation, and speciation. For example, Cox *et al*. (2017) and Ng *et al*. (2013) investigated the effect of environmental and genetic factors on dewlap size and pigmentation with breeding experiments. In addition, by controlling the environment, housing experiments allow scientists to test the effect of specific parameters on a specimen's phenotype or performance. For example, Lailvaux *et al*. (2012) studied the effect of high vs. low food availability on the morphology, dewlap size and bite force of *Anolis carolinensis*. Similarly, Delaney *et al*. (2016) tested whether perch availability affected reproduction in *Anolis sagrei*. Finally, breeding and housing experiments could allow scientists to study phenotypic and developmental plasticity, as the morphology and/or behavior of hatchlings in response to certain conditions can be studied during their ontogeny.

For my current experiment, we plan to raise *Anolis sagrei* hatchlings on different feeding regimes (hard vs soft diet) and different levels of competition (no contact between males, regular contact, and continuous contact) to see how it affects their head shape and feeding performance by measuring several aspects of head shape (head width, height, etc.) and bite force during the development of the hatchlings to adult. This will allow us to directly evaluate whether differences in diet and/or aggressiveness are influencing the frequently observed sexual dimorphism in head shape and size in *Anolis* lizards. To set up lizard room in Ghent, Belgium, I visited the animal care facility in the Losos lab. There, I was guided and assisted by Anthony Geneva, Colin Donihue, Matthew Gage, Cory Hahn and Jeff Breeze, who shared with me their experience establishing and maintaining an *Anolis* lizard breeding colony.

This research visit resulted in this document, which details protocols for establishing, maintaining, and conducting research in an Anole breeding colony and can serve as the basis for

creating new facilities. It gives a detailed and comprehensive overview of the specific requirements for setting up a lizard room. In addition, the document provides guidelines and tips on general lizard room maintenance, cage building, hatchling handling, lizard transportation and identification and cricket housing.

The general design of the animal care room was developed for the care of *Anolis distichus* and *A. sagrei* and some portions of this protocol are most useful to these species. The same procedures and facilities have also been used to house *A. brevirostris*, *A. carolinensis*, *A. extremus*, and *A. grahami*. Modification of the cage design described in this document has been used to house larger bodied anole species such as *A. equestris* and *A. leachii*.

2: Creating an animal facility for Anoles

2.1 Room Design

Facilities requirements:

RO or distilled water

Reverse osmosis (RO) water is ideal for keeping the lizards healthy. Distilled water is an acceptable alternative. The water is used for general cleaning as well as twice daily misting of lizard cages, which provide drinking water for captive anoles and maintains a humid microenvironment.

Lighting

For proper lighting, water-vapor resistant lights (F32T8 fluorescent bulb fixture) should be used. For bulbs, we recommend UV lizard bulbs or, the less expensive, full-spectrum bulbs (32W 6500K). These light bulbs can lose their ability to produce a portion of the UV light spectrum over time and should be replaced annually.

In general, we employ a 14h daylight/10h darkness scheme during breeding season and 12h light/12h dark for winter cycling. Sanger *et al*. (2008) used a 13h light/11h dark during summer, shifting to 11h light /13h dark during winter months (early October – late March). A short winter period (one-two month) might stimulate reproduction. Some species will eat less during this simulated winter and therefore should be fed less often. To ensure complete darkness, windows must be covered.

Temperature and Humidity

We currently maintain room temperature at 84 ± 1° F (28-29° C). For this, a programmable temperature control should be used, ideally with the potential for two settings per day (day and night). Our target for minimum humidity is 40% in the lizard room and minimum of 60% in the cage. A programmable humidity control can be used as well, although high ambient humidity is often difficult to maintain due to air exchange requirements of university vivaria. Our cage design maintains a far more humid microenvironment than ambient room humidity. For most species the higher the

cage humidity, the better (around 80% is optimal). If humidity is too low in the cages, misting more than twice per day may be necessary.

Shelving

The shelves should be made of rust-resistant and UV-resistant material, such as stainless steel with a chrome coating. The space below the bottom shelf is convenient for cricket storage (red arrow in Figure 1), with no light fixture above. The lowest shelf is placed just above the cricket boxes. Each shelf contains several cages next to each other (see picture), with a light installed on the bottom of each. Make sure there is enough space between the lights and the cages_themselves, as water and food are provided through the top of each cage (see "Section 2.2 cage building"). Due to this set-up, a light will be above and below the two upper-most shelves (blue arrow), while only one is present above the cages on the lowest shelf (green arrow). Consequently, the cages on the two uppermost cages can be slightly warmer and less humid then the cages on the lower shelves. To prevent a cage effect in experiments, cages could be switched between each shelf on a regular basis. Alternatively, all different treatments can be put on the lower shelf as well, in order to control for any systematic bias.

Figure 1: Lizard facility shelving units. The three uppermost shelves are used for lizard housing (blue and orange arrow); the cages on the ground floor for cricket storage (red arrow).

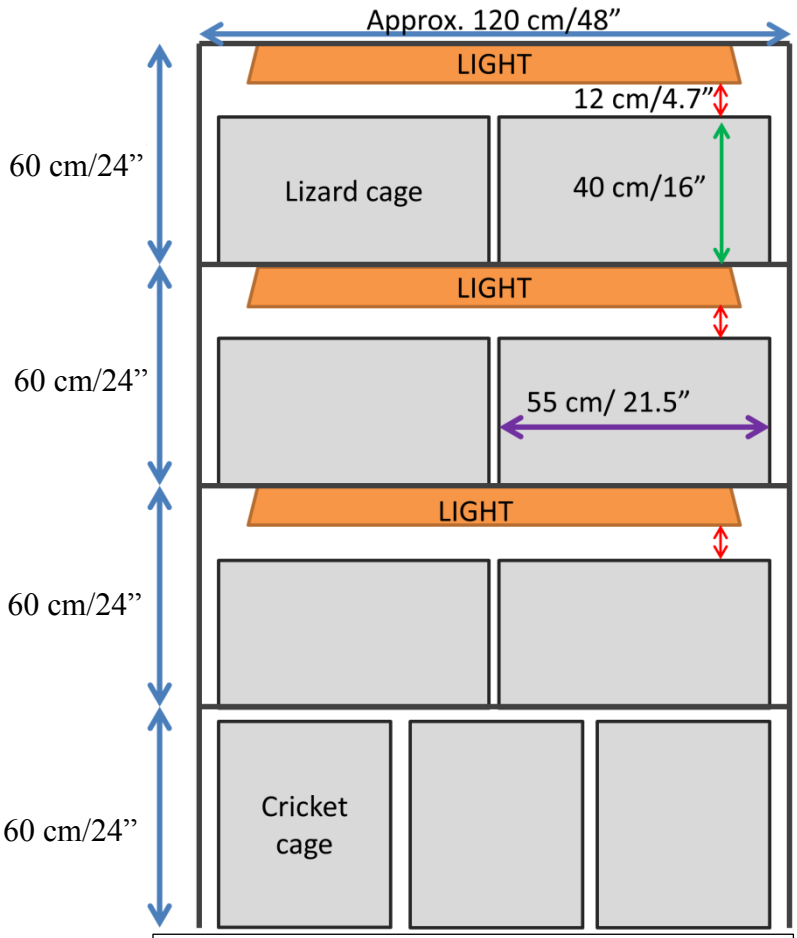


Figure 2: Building scheme for shelves

Work areas

Ideally, the room should have floor drains and moisture resistant floor, wall, and ceiling coverings. It’s useful to have large standing-height countertop space for work areas and any animal care procedures that may arise.

Procedure area

A separate space for cleaning, specimen preparation, and general storage (consisting of shelving units as well as drawers). This room can be situated within the animal care area or an adjoining room. The temperature in this area does not need to be the same as the animal care area. This area requires approximately 10ft of counter space including a deep basin sink with RO, hot, and cold water taps. This space also contains small 4ºC refrigerator and -20ºC and freezer (can be a single unit as long as -20ºC space in not frost-free).

Autoclave

Cage dressings and soil are autoclaved before use. Autoclave access near to the lizard facility is needed, but a dedicated autoclave is not necessary.

Entry

Ideally at least two sets of doors separated the animal care facility and public space, with room access being controlled via card or key if possible. The intervening space

should have storage cabinets and wall hooks for hanging personal items. Doors should have tight seals on all sides, and a sweep underneath to prevent cricket escapes.

Cricket storage

Crickets are stored in separate containers depending on their size (pinheads, ¼ in, 1/8 in and 3/8 in; or in mm: pinheads, 32mm, 64 mm, 96 mm). Containers should be high enough to prevent them from jumping out. Several large holes should be made in the cover of each container (3"/ 7.5 cm diameter). These holes should be covered with a screen to prevent cricket escape. Each container is labeled with cricket size, date of arrival and, if necessary, a box number. We have found it useful to color-code the labels of cricket tubs by size and lizard cages so it is clear which lizards receive which size crickets. These containers are placed on the ground underneath the lowest shelf (see above). For a lizard room of approximately 1000 lizards: consider 38 ft^2 of storage space (12m^2).

Signage

It is recommended to have the most useful files printed out and always visible in the lizard room. These signs include: toe clipping scheme, sexing guide, vermiculite mix, and feeding color labels.

Cage labelling

Cage labels contain the following information: cage number, species, locality, origin (wild-captive), specimen ID, sex, introduction date/date of birth and parental information (if required)

Figure 3: Cricket cage, with holes covered by a screen in order to avoid escape.

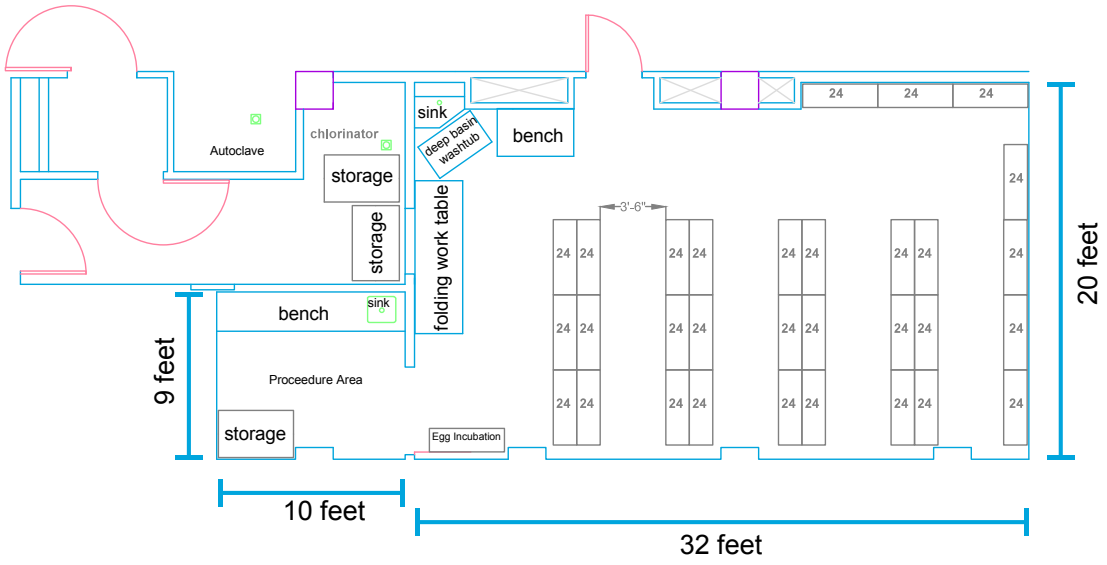


Figure 4: Example of the annotated floor plan of a lizard facility

2.2 Cage building

Materials

Screen

Aluminum mesh screen is used to cover the top of the cage. This allows air exchange and for misting cages without the need of opening them.

Acrylic panels

Cages are constructed from relatively inexpensive, sturdy acrylic panels. Opaque acrylic sheets are used for the sides and bottom (to limit interaction between cages). Transparent acrylic sheets are used for front and top panels.

Acrylic Cement

Sci-Grip thin set acrylic cement (highly volatile, close when not in use).

Magnets

Easy, automatic closing system. Should be large enough as larger lizards might be able to open the cage when magnets are too small. In case this doesn't suffice, you can use two magnets or an additional closing mechanism such as a hook.

Hinges

Used to open the door and upper lid. Normally, 1 hinge for the upper lid and 2 for the door suffice. As each cage will contain 4 enclosures, 12 hinges suffice for one complete cage construction.

Building protocol

1. Connect outer panels with tape (see Figure 5). Make sure they are perfectly perpendicular to each other and edges are lined up!
2. Wear gloves for all gluing steps. Use a syringe and put a small amount of acrylic glue in the corners (Figure 5: blue arrows) and let dry for 1 minute
3. Glue the remaining contact surfaces (Figure 5: Green arrows)

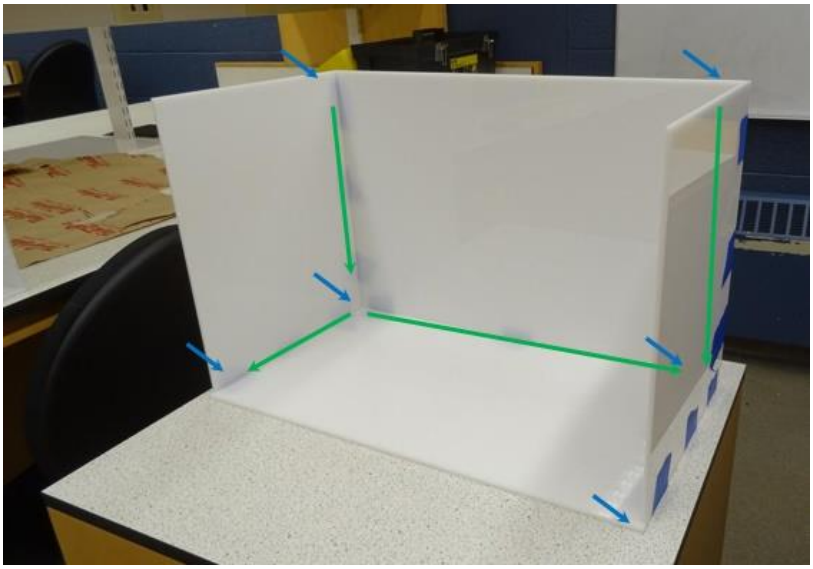


Figure 5

4. Insert cross sections using wooden blocks (use the block on top first, then the one in front). The block on top should be parallel with the acrylic panel at the back.
5. Fix the bottom of each cross section with glue (application to 1 side suffices; Figure 6: blue arrows), subsequently the corners at the upper edges. For gluing these: fist glue the middle cross-section (this makes it easier to press the remaining cross-section panels against the border panel). Add glue through the remaining unglued parts (Figure 6: red arrows). Remove the blocks and glue the parts that were covered by the blocks (Figure 6: orange arrows)

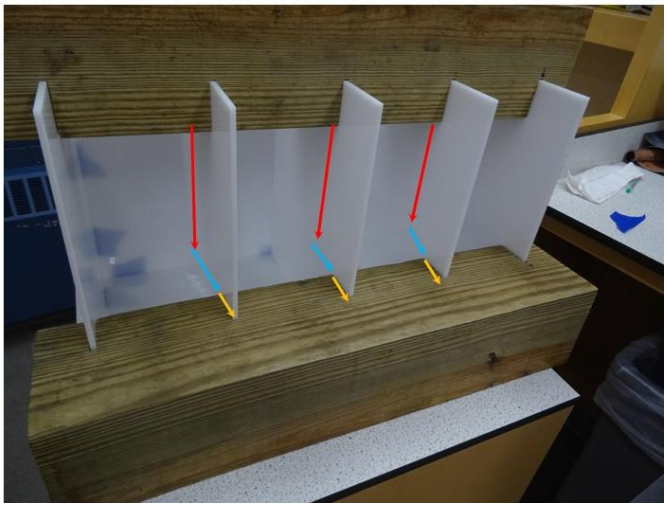


Figure 6

6. Take transparent acrylic parts and sand the sides that will be touched during feeding and cleaning. Wear a mask during this step.
7. Use ethanol to remove dust from the sanded sides.
8. Attach the panels as shown in Figure 7 using tape. Once the panels are stable, glue the lower panels (yellow arrow indicates sanded side) in the following order: First, blue, then

red and finally the panels at the green arrows (Figure 7). Then do the same for the upper panels. Remove tape once the glue is dried.

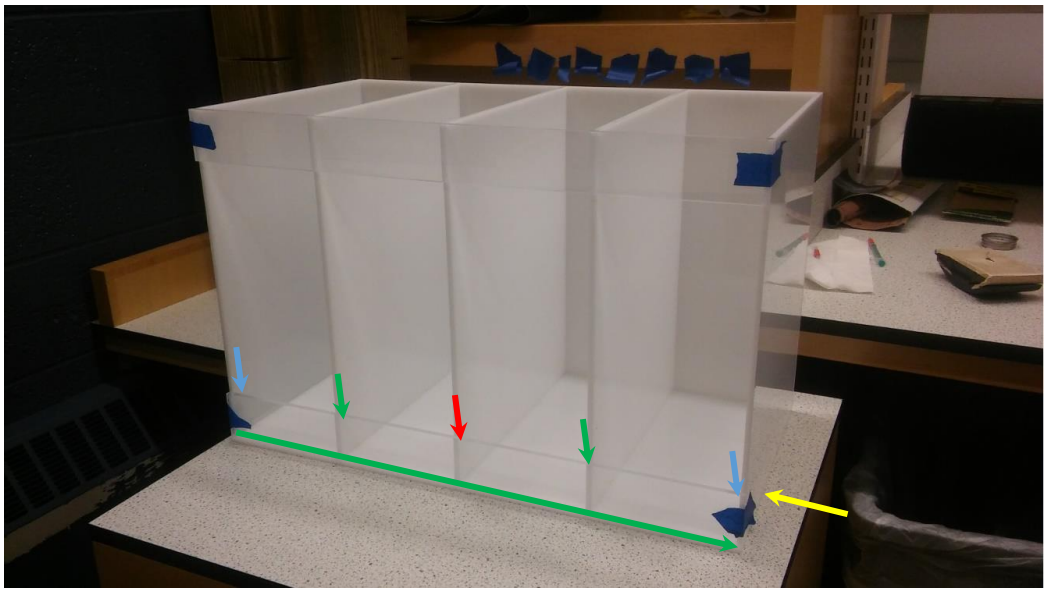


Figure 7

9. Prepare the top of the cage a shown in Figure 8 in the following order: blue, red, green. The panels on the cross-sections (light red) shouldn't be perfectly in the middle, as screen mesh will be covering the openings. Yellow arrow indicates stranded edge. To attach these panels, you will put the syringe upward, so make sure to wear goggles.

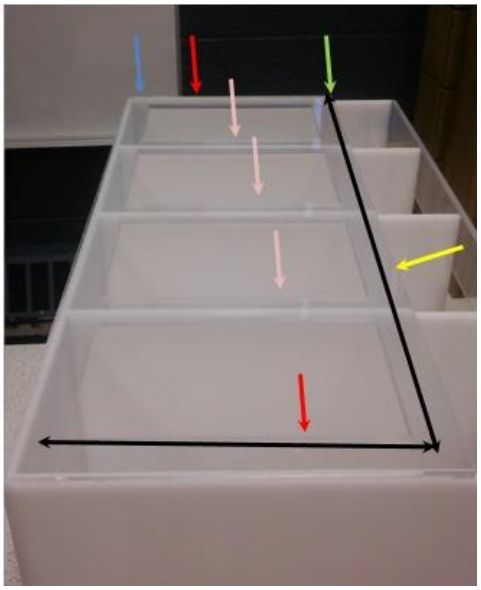


Figure 8

10. Measure the required size of the mesh screen, the screen should normally reach only to half of the transparent upper panels (black arrows in Figure 8). Normally, the size should be 20.5X53.5 cm (8 X 21 in).

11. Cut the screen mesh to the proper size, remove any loose ends.
12. Put screen on upper layer and attach the first transparent panel. Align this panel as best as possible with the one below using tape. Add glue on both sides of the panel (see arrows in Figure 9).

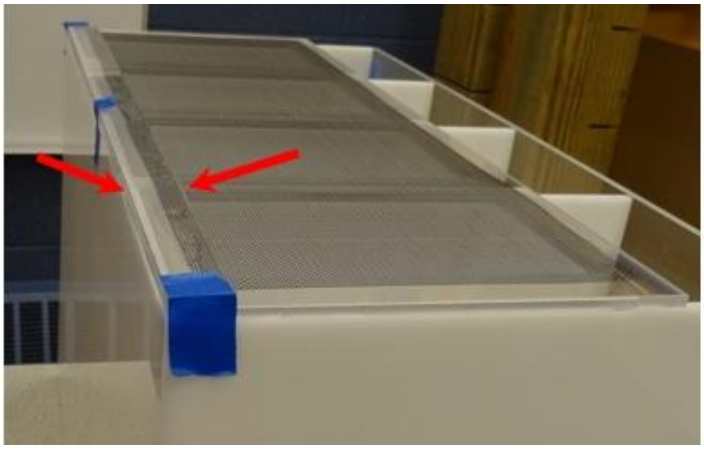


Figure 9

13. Now you can build up the other panels as shown in Figure 8. Make sure all panels are aligned as best as possible and again, make sure the screen mesh has a wet look by using a lot of glue. The screen mesh should only bulge in a little when being touched after gluing. The cage should now look like Figure 10.

Figure 10

14. Put some large, heavy objects on the upper borders to improve glue bonding.
15. While the glue is drying, take the final transparent panels used as "front door" and "upper lid". Sand three sides of these panels (the upper side that won't be touched can be left alone). Don't forget to wear a mask! Clean the panels with ethanol to remove dust.
16. Remove the blocks from the cage and put the upper lids in line with the cross-sections (the sides of the lids should be on the white of the cross-sections). You can also leave a very tiny space between the sides of each of the upper lids (approx. 1 mm), which allows to easily open and close these lids.
17. Put one hinge in the middle of each panel. Now you can glue the hinges to the panels. Only apply glue on the front and the back side of the hinge as shown below (green arrow in Figure 11). If glue would come between the wrong panels (red arrow), immediately open the lid and remove the glue with paper towels. Yellow arrows indicate stranded sides.

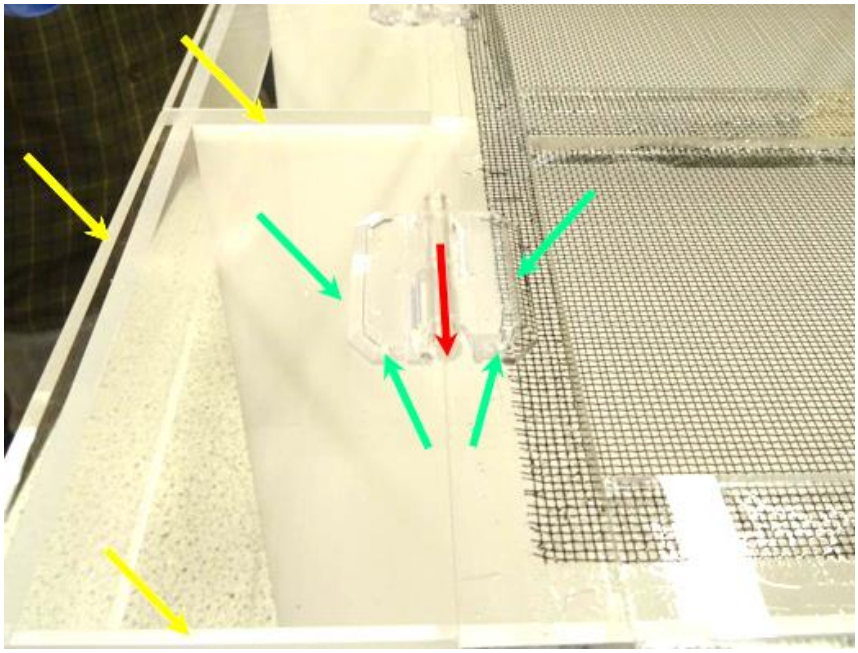


Figure 11

18. Align the four doors and attach them to each other by tape as shown in Figure 12 (makes it easy to move them). You can add more tape if you want. Make sure the sides of each door covers the white edge of a cross-section.

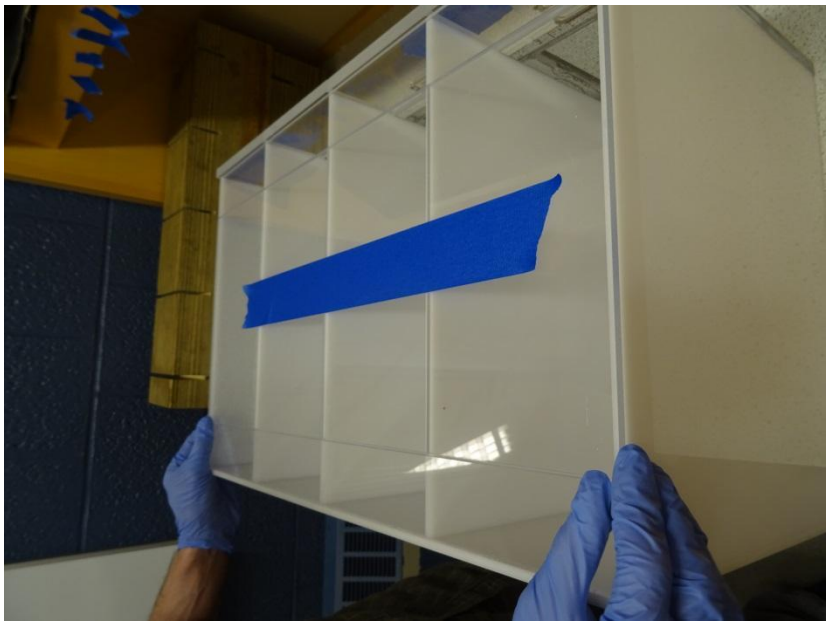


Figure 12

19. Attach two hinges per door as was done in step 17. You can leave a small gap between the door and the lower transparent panel as shown in the figure below (but make sure that a hatchling can't fit through).

20. Make sure your magnets are built as in Figure 13. The rectangular block should make a straight line with the magnet when closed!!

Figure 13

21. Place the upper part of the magnet such that it is in the middle of the door, with the edge with the green arrow above being perfectly in line with the lower edge of the door. Glue the magnet at the position of the green arrows as shown in Figure 14.

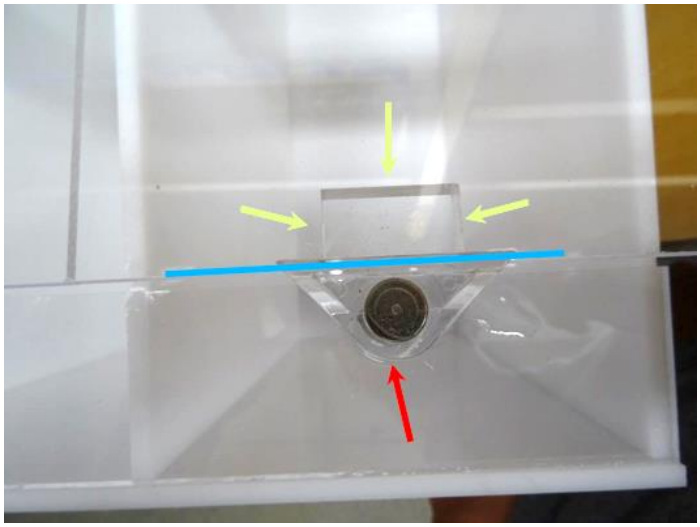


Figure 14

22. Once the glue has dried, prop the doors open to attach the lower part of the magnet.
23. Glue the lower part of the magnet. Make sure the magnet doesn't hang over the edge, so position it as shown below. Put a mark at the edge of the magnet (red arrow in Figure 14 and 15) so you can easily determine its position.

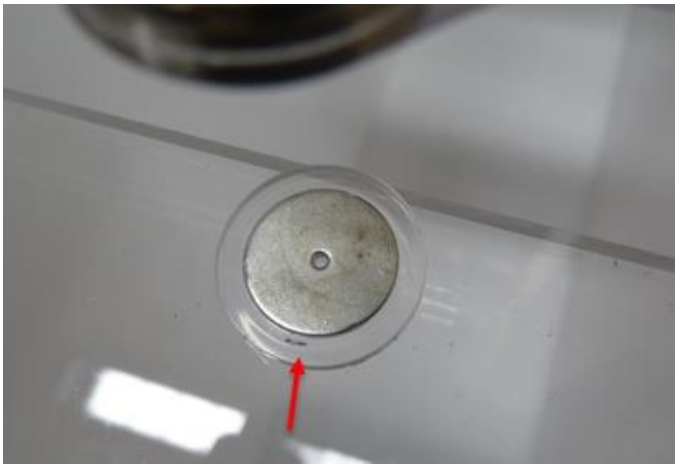


Figure 15

Protocol and hints for building cages (sanding, gluing, etc.)

- Use goggles when applying the acrylic parts on top.
- Make sure all acrylic panels are completely on the shelf (so no parts hanging over edges) as this can warp acrylic panels over the long-term
- Put a piece of cloth or paper underneath the transparent acrylic panels. This prevents them from being scratched when moved, which reduces visibility.
- Use all-plastic syringes. Rubber gaskets are dissolved by acrylic cement.
- If glue accidentally drips on the transparent panels allow it to dry in place. Do NOT try to remove this as it will make a blurry stain on the panels, impairing visibility.

2.3 Cage dressings

Soil

Use "Organic potting mix" as this does not contain any added fertilizer. Autoclave all soil before use. Add 475 cc soil per cage, which provides a thin layer covering the base of the cage which helps maintain humidity but is too shallow for egg laying. Soil should only be changed every three months as this is stressful for the lizards.

Dowels - Preparation (remove adhesive)

Dowels should be similar in size and diameter (0.5 in/ 1 cm) in each cage, as differences in dowel size can cause a plastic response in limb size (Losos et al., 2000). Dowels should be placed across each other to form and "X". Dowels are sanded to remove sharp edges and adhesive labels.

Plants

Plastic foliage can be used for each cage. These can be reused and cleaned by soaking in 10% bleach solution for 30 minutes followed by thorough rinsing with RO water.

Egg cups

Polypropylene yogurt cups (1 liter) are used as egg cups. A small hole (diameter: approx. 1 in or 2-3 cm) is made in the lid of these cups where females can dig in and lay their egg. The cups are completely filled with a vermiculite-water mix (18:11 water to vermiculite by weight). Label each cup with the cage number. If the eggs are being laid in the soil instead of the egg cup, this indicates that the mixture in the egg cup is either too dry or too moist. Replace or remoisten the vermiculite mix in this case.

Figure 16: Completed cage with cage dressings

3: Establishing an Anole colony

3.1 Collecting animals from the field

In field

Butterfly cage

Females can be put together in a large butterfly cage. This cage can easily be sprayed so that the lizards have water.

Cloth bags

Males should be kept separate. For this, you can use a cloth bag with a string that allows you to close it. You can spray these bags so that the lizards have water.

Ice Chests

Put the butterfly cage/cloth bags in an ice chest to move the lizards from the field to your place of stay. Make sure that the size of the ice chest is suitable for the number of lizards you want to transport. Also check the isolating capability of the chest, the more it holds the temperature the better.

Thermal Blocks

Thermal blocks (phase change material) are used to buffer the temperature in the ice chest from extreme highs and lows. The example shown below holds the temperature at 22°C. You can put the thermal blocks outside of the chest while working in the field.

Figure 17: Thermal block used to keep a constant temperature in the ice chest

Padding

Use cloths or other soft material to steady the cages and thermal blocks.

Moving the lizards

Individual Containers

For moving, the lizards are transferred from the cloth bags/butterfly cage to individual containers. We use egg incubation cups for this (for picture, see "Section 4.4 Egg check"). Place a sheet of soft paper in the cup and lightly spray. Also spray the sides of the cup so that the lizard has enough water. Lizards can live over a week without food, so no food needs to be supplied. Put the cups into the ice chest, together with the thermal blocks and proper padding.

3.2 Anole Biology in Captivity

Sperm storage

Female lizards can store sperm (and thus lay eggs) for >4 months on average after copulation. This has the following consequences:

- If females are collected from the field, sexes should be kept separate initially. If you put males and females together before that, paternity with be uncertain. Provide egg laying cups in female cages and check for eggs from wild-caught females. When the ratio infertile/fertile eggs becomes large, you can introduce the male (for genetic experiments, a maximum 1% of the eggs should be fertile; for a non-genetic experiment, the ratio can be higher, but introduces error).
- To start breeding lizards, males should only be put together for a short period (48h – 1 week), after this, the female can reliably lay eggs for 2-4 months.

Reproductive period

West Indian anoles in nature lay eggs from about March through October. Wild-caught females keep following this rhythm even when brought to the lab. Lab-bred lizards are less prone to this. However, both for wild-caught and lab-bred lizards, it is good to induce a winter period of 6 to 8 weeks. For this, the temperature should be dropped by minimum 2° and the light-dark period should be shifted so it is longer dark than light (see also "Lighting" in Section 2.1). After returning to summer, wait 1-2 weeks before establishing breeding pairs.

Generation time

The generation time differs between males and females and among species. In *A. sagrei* females are usually reproductive 6 months after hatching. For males, this is approximately 7-8 months. Once the male develops secondary sexual characteristics, they can be used for breeding.

Aggression (M-M, M-F, F-F)

- General: Hatchlings are housed together if they are born within the same week.
- Male-male aggression: In our experience, males are normally not aggressive if they grew up together. However, aggression might be observed if A) there is not enough food or/and B) a female is visible (e.g. in the cages on the opposing side). In this case, males should be separated. Also make sure that the males that are put together are similar in size.
- Male-female aggression: Breeding pairs can generally be kept together. However, make sure to check the health of the female regularly. While there might not be bite wounds, she might still be stressed.
- Female-female aggression: We have only occasionally observed female-female aggression. If bite wounds are observed, separate the females.

Pairing animals (considerations and best practices)

- Introduce females first and let them acclimate for a week before introducing males.
- In many cases mating occurs immediately upon introduction.
- While normally 6 adult crickets suffice per lizard, this amount should be ~2.5 times as much when pairing animals as males eat substantially more and no food would remain for the females. When feeding breeding pairs, only a few crickets should remain after 2 days. In case all crickets are gone, supply more crickets.

3.3: Setting up cages

Sex segregation

- Males and females can be readily distinguished about 1 month after hatching (see "Section 4.7 Handling cohorts")
- You can keep up to 3 males together in one cage (regularly check for bite wounds). If males grew up in the same cage, they should not interact with each other. Possible interaction might be caused by the visibility of a female or by food shortage.
- Up to four females can be kept in one cage if they are not laying eggs. If females are egg laying, keep them separate.
- There should only be 1 breeding pair per cage.
- The above amounts are appropriate for smaller lizards (*A. sagrei, distichus*, etc.). Larger lizards (such as crown giants) should be kept individually.

Identification: toe clipping

Toe clipping allows for the unique identification of up to 9999 animals (see Appendix for clipping scheme). This protocol results in no more than two toes to be removed from any one extremity and also ensures that adjacent toes are never removed. Toes should be removed at the base of the knuckle using heat-sterilized micro dissecting spring scissors.

4: Maintaining a breeding colony

4.1 Cricket care and maintenance

Cricket ordering

- Check the current stock of crickets before ordering new crickets in order to determine the required amount of new crickets
- When new boxes of crickets arrive, open them immediately and empty each box of crickets into a new plastic bin. Remove any excess paper or cardboard.

Cricket food & water

- Orange cricket cubes (red arrow in Figure 18): enhance the nutritive value of crickets by supplementing calcium and other vitamins. Administer a few of these cubes to the crickets.
- Chicken feed pellet (blue arrow): Add one dose (covers approx. one corner of the box)
- Sweet potato (completely eaten in picture): cut into small pieces and put a few of those on top of the chicken feed.
- Use water crystals (soil moisture granules; green arrow) to provide water to the crickets. This prevents the crickets from drowning. Cover the bottom of a cup and fill it with water, the crystals will absorb the water in a minute. Subsequently, place a lid with a 1cm layer of moistened water crystals in the box. Make sure the water crystals are not overflowing and coming into contact with the food or egg crates.
- Check cricket food & water regularly (each 2-3 days). Add food if this is finished. You can just put new wet crystals over the older crystals when dried (crystals are dried in Figure 18)

Setting up cricket boxes

- For built-up, see Figure 18. Up to 3 egg crates can be kept in the box on one side of the bin.
- Use as many crickets as possible in each box. Around 6000 crickets can be kept per container (pinheads, 1/8" and 3/8") and around 5000 per cage for the largest crickets (1/4").
- Cricket die offs can occur as a result of excess moisture in the cricket bins.

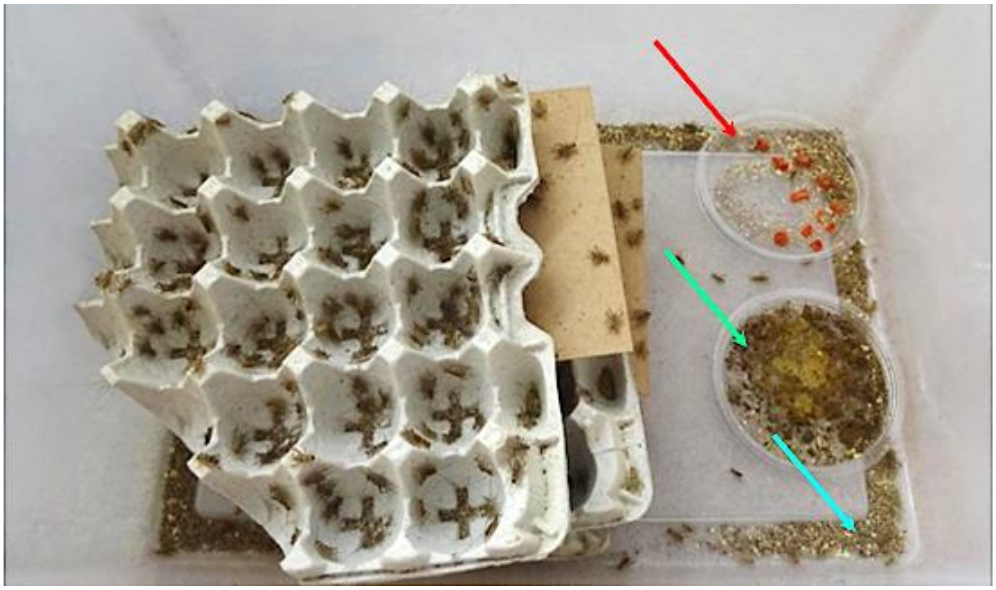


Figure 18: Cage dressing in cricket box

Cleaning cricket boxes

Discard food, egg crates, and water crystals. Rinse the box in the floor sink with hot water to remove as much material as possible, then soak with hot water and dish detergent for at least 10 minutes (and up to overnight). After soaking, wipe down walls with sudsy water then rinse with RO water and dry completely before reusing.

Escaped crickets and other pest arthropods (mites, spiders, etc.)

Crickets may escape while feeding the lizards. In addition, other insects and spiders may be present in the room. Therefore, 8 – 12 cricket traps are placed around the room at least twice a week. The date when the trap is placed is mentioned on each trap. A color code can be used for traps that are placed on the same day. Make sure to also put traps just outside the room for crickets or other insects that might escape. IMPORTANT: Escaped lizards can get stuck in these traps. Therefore, it is important to close of the openings of the traps with some tape as shown in Figure 19 This way, the opening of the trap is still large enough for insects to enter, but too small for lizards.

Figure 19: Entrance to cricket trap is partially blocked in order to avoid lizards getting stuck.

4.2 Feeding Procedures

How to prepare the feed (+ tips & tricks)

- To ensure that the lizards obtain enough vitamins, vitamin D and calcium, these are added to the cricket diet. Fill a separate cup with vitamin mix (50%) and vitaminD+Ca mix (50%). The vitaminD+Ca mix should be phosphorus-free! If excess bone growth is observed in lizards, reduce the frequency of calcium supplementation.
- Crickets can be collected most easily by emptying the egg crates in a separate bucket (for pinheads, this can be immediately in a small deli cup, for larger crickets use a flexible bucket that is high enough so crickets cannot escape. When removing the egg crates, be sure to prevent that the egg crates come into contact with the water crystals.
- Dust the pinheads and crickets with the vitamin-mixture
- Use the flexible bucket as a funnel to put the dusted larger crickets in a smaller deli cup. Fill this cup to around 1/3 its depth (more might cause the crickets to die before being given to the lizards)
- The crickets can now be given to the lizards

How to add lizards in cage

- Before lifting the cage lid, make sure that no lizards are near to prevent escape. In case lizards are close to the lid, tap the cage so they move away.
- In case any dead animal is present in the cage, remove it prior to feeding.
- Use the deli cup to drop the crickets into the cage.
- Before closing the lid, make sure the tail of the lizard will not be crushed! Sometimes, it is better to let the lizard escape than to hurt it by dropping the lid.

How many crickets and how often

- Hatchlings are fed daily. They can get 6-12 pinheads per individual. All (or at least most) pinheads should be eaten the day after. If there are still pinheads present, give a lower dose (pinheads can bite and wound hatchlings)
- For older/larger lizards: give crickets appropriate for their head size. If the cricket fits their mouth, they will eat it. These lizards are fed 6-12 adult crickets per lizard 3 times a week. Make sure to check whether most crickets are gone from previous feeding. While this level of feeding is appropriate for *Anolis sagrei,* differently sized species may need more or fewer crickets.
- If lizards are hungry, they start eating immediately.

Cage labeling

Feeding is simplified if each cage is labeled with a color that corresponds to the cricket size that the lizards should get. The labels on the cricket storage boxes should match this color.

Drosophila for hatchlings:

When a cricket shipment goes wrong, it is especially important that the hatchlings get their food. One can hold a *Drosophila* population as a reserve in case this happens.

4.2 Animal health check

- Feeding time is the perfect opportunity to check on the health of all animals. When providing food, be sure to check that all animals are present and healthy.
- Following signs indicate that the lizard may have health issues:
  1. Lizard is laying on its back
  2. Lizard is sitting on the soil
  3. Lizard has a darker color (stress)
  4. Lizard has been eating soil (will die very soon)
  5. Visible tumor growth
- Check older animals for bite wounds: female bite wounds often indicate a shortage of food; male bite wounds might indicate that there is a shortage of food or that they can observe females
- Sick animals can be placed in separate bins with paper on the bottom. In case necessary, the animals can be fed by hand.

4.3 Misting

Setup and equipment

MANUALLY: hose and a nozzle with multiple settings (should include mist-setting). Taps for daily spraying must be able to remain open without being held.

AUTOMATIC: Large basin from which water can be pumped. Automatic misting system, including timer, pump, hose and a nozzle for each cage. Make sure the nozzle points downward towards each cage. Depending on the number of cages, multiple pumps should be used.

RO water

Use RO or distilled water to spray the cages. Set the nozzle to the "mist"-setting and try to spray on each of the walls of the cage, while avoiding to saturate the soil.

Frequency

Each cage should be sprayed twice a day. Once before 10 AM and once after 4 PM. If the humidity in the cages is too low, spray more frequently.

4.4 Egg checks

Division of labor

Depending on the amount of eggs, the work can be subdivided among different people. First, collect all the egg cups. Subsequently, one or two people empty the egg cups in a separate bin and check the vermiculite for eggs. In case an egg is present, it is moved to an incubation cup which should be labelled immediately. Used vermiculite is thrown away. A third person subsequently cleans the empty yogurt cups. Once cleaned, one or two people refill the egg cups with the vermiculite mix. Finally, one person is responsible for putting the egg cups back in the right cage.

Making egg incubation cups

Incubation cups can be made in transparent, plastic boxes as shown in the picture below. Fill each cup with a 130g vermiculite/water mix (18:11 water to vermiculite by weight). Eggs should be put on top of the vermiculite/water mix. In case not present, make small holes (appr; 2-3 mm) in the cup to provide oxygen. Close the cups once finished.

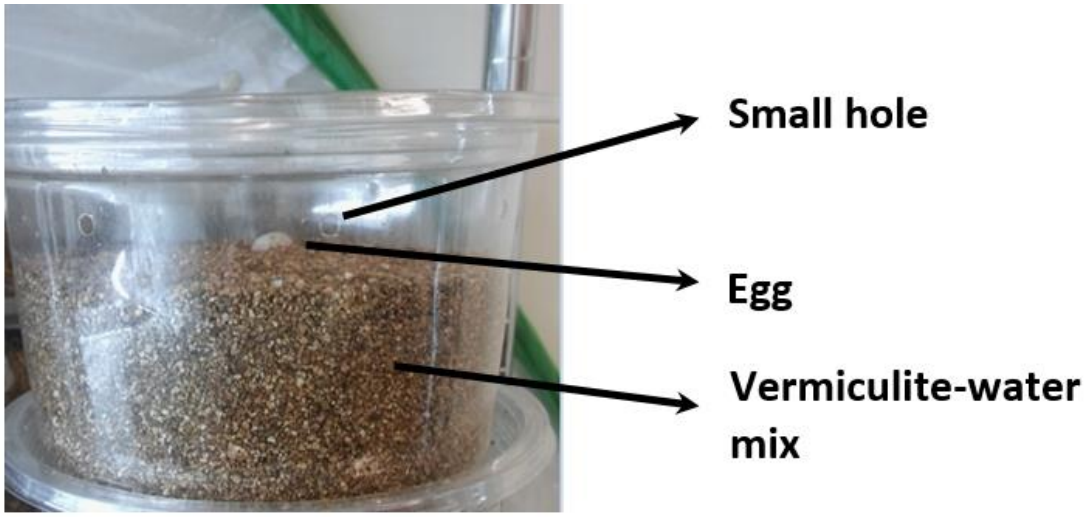


Figure 20: Template for egg incubation cup

Labels

Each egg is labeled with a specific number (which will also be the ID of the hatchling). Put this number both on the lid as the cup itself. This way, eggs can be linked to their origin (see "Record keeping on fertile and infertile eggs").

Distinguishing fertile and infertile eggs

Infertile eggs are typically yellow, small and uncalcified, whereas fertile eggs are white, large and calcified (see Figure 21).

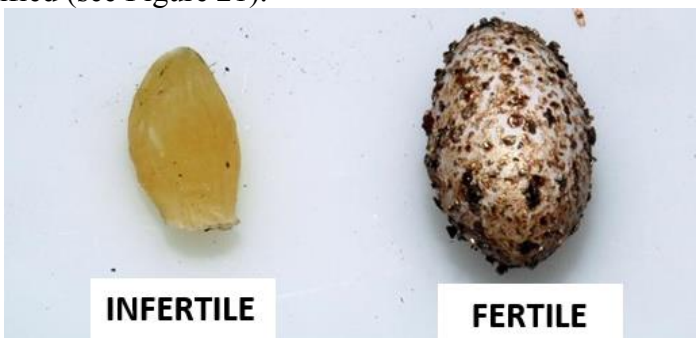


Figure 21: Infertile vs fertile lizard egg

Cleaning, checking, rehydrating egg laying cups (yogurt cups)

Gloves should be worn and washed or changed throughout egg checks to prevent disease spread among cages. The yogurt cups are checked once each week. All vermiculite should be sorted and rehydrated if too dry. Wash egg cup and lid before returning the cup to the cage. If a female is laying her eggs on top of the yogurt cup or in the soil, this usually indicates that there is something wrong with the vermiculite mix (either too dry or too moist). In that case, remove the yogurt cup and replace the vermiculite.

Record keeping on fertile and infertile eggs

The relative production of fertile and infertile eggs is often informative, and critical data for measures of reproductive isolation. Make sure that in your record keeping file contains a column in which the state of the egg can be mentioned: Y (Yellow or Infertile) or W (White or fertile). Keep track of the amount of infertile eggs compared to fertile eggs.

Animal record keeping: Paper and digital

Make sure both a hard copy with all information of the egg/hatchling and a digital version of this info are present

4.5 Managing incubating eggs

Considerations and best practices

- Check incubating eggs daily for hatchlings or failed incubations. It takes approximately 1 month for *Anolis sagrei* eggs to hatch.
- If a dent is present in the egg, this indicates that the vermiculite mix was made incorrectly (too dry). Record the dent and mist or add a drop of water to dented eggs to try to restore it.
- Just before hatching, condensation might be observed on the egg. This is commonly called "sweating".

4.6 Newborn lizards

Introduction of newborns

- Check the egg cup daily for new hatchlings, they will often be running on top of the vermiculite mix.
- Provide an empty incubation cup with folded, moistened paper towel. Transfer the hatchling to the new cup and leave the hatchling there for a few minutes, so it can remove vermiculite that is attached to its body.
- After ~5 minutes, you can grab the hatchling gently (best to hold it at its thigh) and remove remaining vermiculite around the eye or body with a paper towel.
- Weigh the hatchling and report its weight in the data log.
- Then toe clip following the toe clipping scheme posted in the lizard room (see Appendix).

Tips and remarks

- Hatchlings are easily stressed due to excessive handling. If toe clipping takes too long, one might put the hatchling back in the cup for a few minutes so it can relax again.
- Try not to hold the hatchling at its body cavity, they might overheat! Again, holding them around the thigh is the best strategy.
- For toe clipping, either use your bare hands or gloves that are one size too small so they are stretched. This makes it easier to spread the toes for toe clipping. In case using your bare hands, do not forget to wash these first!
- Remember: lizards might play dead. In that case, also put them back in the cup to relax them again.

4.7 Handling cohorts

Sexing juveniles

Male *Anolis sagrei* develop a dewlap around 3 months of age. Determining the sex of lizards can be complicated for young animals. The most reliable method to determine the sex of an animal is the presence or absence of enlarged post-anal scales. Males have two enlarged scales a few rows below their cloaca whereas females will have more or less evenly sized scales in each row. Even hatchlings will show a difference in this trait when observed under a microscope. The scales become more easily observable when the lizards get older. The post-anal scales of males should be easily visible by scope after 1 month.

Grouping of hatchlings, juveniles and adults

- HATCHLINGS: 4-6 hatchlings can be kept together in one cage, as long as they are born in the same week.
- JUVENILES and especially ADULTS should be separated by size and sex. A cage can contain either 3 males, 4 females or 1 breeding pair. Make sure that the lizards in one cage are the same size.

4.8 Cage Cleaning

Cages are generally cleaned as follows:

- Remove enrichment: plastic plants and dowels can be kept in a separate cleaning bin.
- Vacuum the cage to remove soil.
- Rinse the cage with a bleach solution (10% bleach – 90% water).
- Using a paper towels remove any all traces of dirt and feces from cage.

- Rinse cage multiple times with RO water to remove residual bleach and let the cage dry for 24 hours.
- For the plastic plants and dowels: fill a basin with a 10% bleach solution and put the material in the basin. Keep it in there for a couple of hours. Then remove the solution and rinse the material several times with water. This material can be reused once dried.

4.9 Managing Lizard Issues

Escapes

- All walls, ceiling, and spaces under cabinetry must be sealed to prevent lizards or crickets from escaping. All air vents and floor drains should be covered with fine mesh screening. Even small holes should be covered as hatchlings might get in these.
- It might be better to let a lizard escape rather than closing the lid quickly as the lizard might get stuck between the cage and closing lid, causing large wounds.
- In case a lizard escapes, try to capture it immediately!

Disease

- In case a lizard is sick (see also "Section 4.2 Animal health check"), you should separate it from the other lizards.
- As long as a lizard is able to feed, there should be no problem. As the lizard is no longer able to feed, you may try to hand feed using a syringe.
- In case the animal is too sick, one might want to contact the local vet or euthanize the animal (See section "5.4 Euthanasia and preparing museum specimens").

Mortality

- It is critically important that you properly identify and label dead animals. It is very difficult to undo mistakes made at this stage!
- FAILED EGGS: See "Section 4.5 Managing incubating eggs"
- HATCHLING: Remove hatchling from cage. Record death date in data log. Store in 2 mL tube with hatchling number labeled on the outside in sharpie and inside the tube in pencil on a piece of paper.
- DEAD JUVENILES/ADULTS: Carefully identify animals using toe clipping. If there is any ambiguity in the toe clipping due to decay, identify every other animal remaining in the cage and determine the number of the dead animal by elimination. Using a single line to cross off the dead animal from the cage, leaving the information legible. Enter death data for each animal in the binder. Remove ½ of the tail and put it in 2 mL tube filled with 100% Ethanol with the specimen ID on the outside of the tape and inside the tube on a piece of paper, written in pencil. The rest of the dead animal should be placed in a ziplock bag and placed in the refrigerator. Using a sharpie writing the following information on the outside of the bag: Specimen number, Death date e.g. (05 July 2016) and your initials.

5: Protocols for research in a colony

5.1: Establishing an appropriate block design

Different shelves often have different temperature/humidity conditions

As mentioned in "Section 2.1 Room set-up" under "Shelving", different shelves might have different conditions. The upper shelves will have a light both above and below, whereas cages on the lower shelf only have a light above. Consequently, cages on the lower shelf could have a lower temperature and higher humidity. This could lead to a cage effect. It is

possible to change the cages between the different shelves regularly, but this is unhandy and stressful for the lizards. A proper block design allows us to take into account a potential cage effect. An example of a proper design (3 different conditions) is shown below.

	CAGE 1	CAGE 2	CAGE 3	CAGE 4	CAGE 5	CAGE 6	CAGE 7	CAGE 8
ROW 1	Cond 1	Cond 2	Cond 3	Cond 1	Cond 2	Cond 3	Cond 1	Cond 3
ROW 2	Cond 3	Cond 1	Cond 2	Cond 3	Cond 1	Cond 2	Cond 3	Cond 1
ROW 3	Cond 2	Cond 3	Cond 1	Cond 2	Cond 3	Cond 1	Cond 2	Cond 3

6: Record keeping and common room-wide metrics for calculation

6.1: Updating online records

As mentioned before, each new hatching, egg find or death should be immediately noted on a hard copy file. It is, however, important to update this information in the online-file as well. Do this at least once a week, in order to have a backup when one of both files goes missing.

6.2: Consistent identifiers

To make identification easier, each new egg immediately gets an ID and keeps this ID throughout its whole life. This makes it easy to track all information related to a certain specimen.

6.3: Counts of fertile and infertile eggs

As mentioned previously, it is very useful to determine the amount of yellow eggs vs white eggs. This ratio can be used as a proxy for the fertility of a female. Once the yellow egg/white egg ratio becomes low, a male should be introduced. Furthermore, for genetic experiments, one should wait for the yellow egg/white egg ratio to be very high. When approximately 99% of the eggs are infertile, a male can be introduced with a very high chance that the fertile eggs produced by the female afterwards stemming from the introduced male.

6.4: Survival etc.

Two interesting metrics to determine are “incubation period” and “survival period”. The incubation period can generally be calculated by “date of egg find – date of hatching”. However, remember that this only an approximation, as egg cups are only checked once a week for new eggs. Survival period, on the other hand, can be determined by “Date of death – Date of hatching”.

References

Cox, R.M., Costello, R.A., Camber, B.E. & McGlothlin, J.W. (2017). Multivariate Genetic Architecture of the *Anolis* Dewlap Reveals Both Shared and Sex-specific Features of a Sexual Dimorphic Ornament. *Journal of Evolutionary Biology* **30**: 1262-1275.

Delaney, D.M., Lovern, M.B., & Warner, D.A. (2016). Does Reduced Perch Availability Affect Reproduction in the Brown Anole? An Experimental Test in the Laboratory. *Journal of Herpetology* **50**: 227-232.

Ferner, J.W. (2007) A review of marking techniques for amphibians and reptiles. *Herpetological Circular.* Society for the Study of Amphibians and Reptiles.

Huey, R. B., Hertz, P.E. & Sinervo, B. (2003). Behavioral Drive Versus Behavioral Inertia in Evolution: A Null Model Approach. *American Naturalist* **161**:357–366R

Lailvaux, S.P., Gilbert, R.L. & Edwards, J.R. (2012). A Performance-based Cost to Honest Signaling in Male Green Anole Lizards (*Anolis carolinensis*). *Proceedings of the Royal Society B* **279**: 2841-2848.

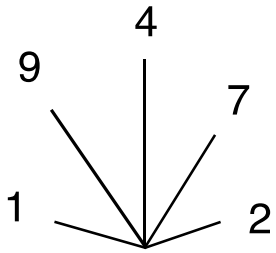
Losos, J.B. (2011). Lizards in an evolutionary tree. Ecology and Adaptive Radiation of Anoles. University of California Press, 528 pp.

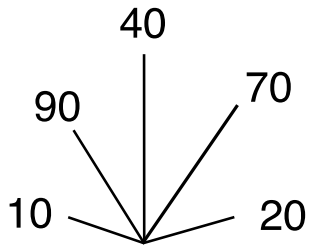
Losos, J.B., Creer, D.A., Glossip, D., Goellner, R., Hampton, A., Roberts, G., Haskell, N., Taylor, P. & Ettling, J. (2000). Evolutionary implications of phenotypic plasticity in the hindlimb of the lizard *Anolis sagrei*. *Evolution* **54**: 301-305.

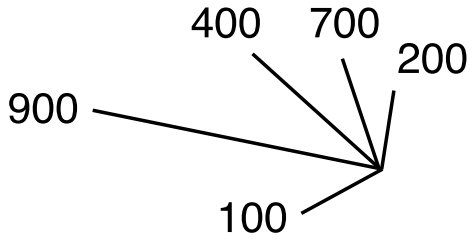
Ng, J., Kelly, A.L., MacGuigan, D.J. & Glor, R.E. (2013). The Role of Heritable and Dietary Factors in the Sexual Signal of a Hispaniolan *Anolis* Lizard, *Anolis distichus*. *Journal of Herpetology* **104**: 862-873

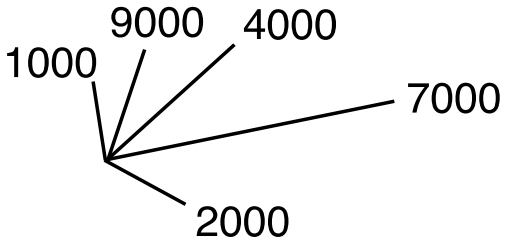
Sanger, T.J., Hime, P.M., Johnson, M.A. & Diani J. (2008). Laboratory Protocols for Husbandry and Embryo Collection of *Anolis* lizards. *Herpetological Review* **39**: 58-63.

Toe clipping scheme (modified from Ferner 2007)









Appendix: Size of cage components (in inches)

Part name	color	thickness	Length	width	quantity per unit	100 units	200 units
Lid long	clear	0.25	22	1	4	400	800
Lid short	clear	0.25	7	1	10	1000	2000
Trap Door	clear	0.5	5.5	3	4	400	800
Bottom	white	0.25	22	12	1	100	200
Back	white	0.25	22	14	1	100	200
Sides	white	0.25	14	11.5	5	500	1000
Anchor/Dam	clear	0.25	22	2	2	200	400
Doors	clear	0.25	9.875	5.5	4	400	800
door knobs	clear	0.5	1	0.5	4	400	800
trap door knobs	clear	0.25	1	0.5	4	400	800