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Protocols for Husbandry and Embryo Collection of a Parthenogenetic Gecko, *Lepidodactylus lugubris* (Squamata: Gekkonidae)

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Lizards and snakes (squamate reptiles) have become increasingly used in developmental biology research, resulting in the establishment of several "model" lizard clades or species (e.g., Sanger et al. 2008; McLean and Vickaryous 2011; Diaz et al. 2017; Infante et al. 2018; Londono et al. 2017; Sanger and Kircher 2017). When choosing a species or clade to study for developmental questions, several criteria must be met. First, a species or group of species must be identified which exhibit the genotype or phenotype of interest. Second, practical criteria must also be considered. The species or group of species must be available for experimentation or observation in the laboratory. Preferably, the species would be available to purchase or easy to obtain from wild populations, easily housed in a laboratory setting with standardized husbandry protocols, have a high fecundity, and have additional resources for investigating developmental questions, such as a sequenced genome or transcriptomes.

The gecko *bauplan* (overall collection of morphological features) is largely considered plesiomorphic (similar to the ancestral form) among squamates (Conrad 2008). Yet geckos also exhibit extremely derived morphologies, such as numerous independent evolutions of adhesive toe pads (e.g., Gamble et al. 2012; Russell et al. 2015). This combination of morphological conservation and novelty, as well as geckos' utility as a system to study convergent evolution (Gamble et al. 2012, 2015a, 2015b), makes them an excellent model clade for developmental evolutionary biology. Their phylogenetic position as the sister clade to all other lizards and snakes, with the possible exception of the Dibamidae (Zheng and Wiens 2016), means that evo-devo studies that include a gecko and almost any other lizard or snake species will have encompassed the phylogenetic breadth of all squamates.

Of more than 1700 described gecko species (Uetz et al. 2017), Leopard Geckos (*Eublepharis macularius*) and Madagascar Ground Geckos (*Paroedura picta*) have been used to study developmental questions (Noro et al. 2009; McLean and Vickaryous 2011). However, another gecko species stands out as an ideal model to study developmental questions, the Mourning Gecko (*Lepidodactylus lugubris*). *Lepidodactylus lugubris* is small-bodied (approximately 40–44 mm snout–vent

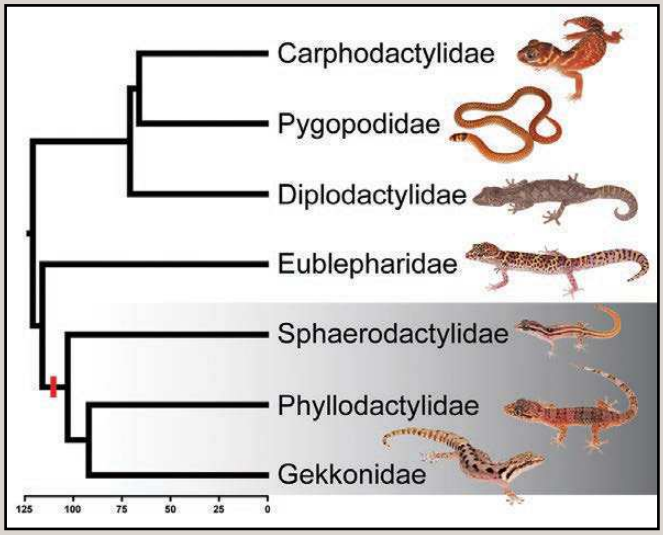

FIG. 1. Phylogenetic relationships of gekkotan families. Red mark indicates the evolution of hard-shelled eggs. Chronogram is scaled to millions of years and modified from Gamble et al. (2015b).

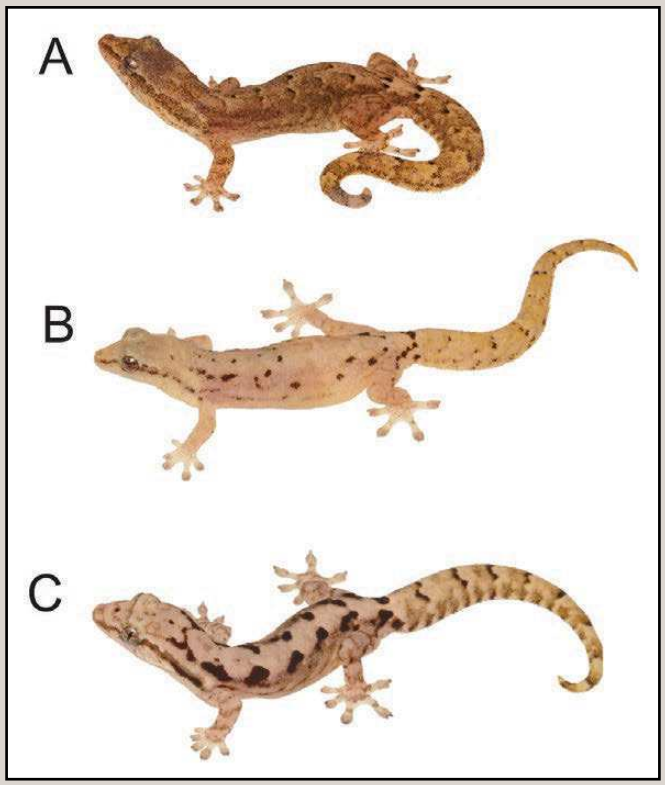

FIG. 2. Color patterns of typical laboratory lineages of *Lepidodactylus lugubris*. A) Clone A, B) Clone B (speckled lineage), C) Clone B (spotted lineage).

length [SVL; Röll 2002]), with a widespread native distribution (India, Sri Lanka, southeast Asia, Indonesia, the Philippines, and nearly all Pacific islands, including the Hawaiian Islands; Bauer and Henle 1994) and multiple introduced populations in the Neotropics (Florida, USA; Central America; northern South America; and the Galapagos; Schauenberg 1968; Henderson et al. 1976; Hoogmoed 1989; Krysko et al. 2011; Hoogmoed and Avila-Pires 2015). *Lepidodactylus lugubris* is parthenogenetic, that is, an all-female species that reproduces in the absence of males, with mothers producing genetically identical daughters (Cuellar and Kluge 1972). Several *L. lugubris* clonal lineages have been described and each is thought to derive from a unique hybridization event between *Lepidodactylus moestus* and an as of yet undescribed *Lepidodactylus* species (Radtkey et al. 1995). Occasional backcrosses between the diploid *L. lugubris* ($2n$=44) and one of the parental species results in triploid *L. lugubris* clones ($3n$=66; Moritz et al. 1993; Radtkey et al. 1995). Between five and 16 clonal lineages have been described (Ineich 1988; Moritz et al. 1993; Yamashiro et al. 2000; Ineich 2015) based on dorsal pattern variation, karyotypes, and allozyme variation (Ineich 1988; Moritz et al. 1993). While the exact mechanisms of reproduction in *L. lugubris* remain unknown, in other parthenogenetic lizards the number of chromosomes doubles prior to meiosis leading to mature diploid oocytes (Darevsky et al. 1985; Lutes et al. 2010). Although *L. lugubris* is parthenogenetic, male phenotypes are occasionally encountered in the wild and in captivity (Schauenberg 1968; Cuellar and Kluge 1972; Ineich and Ota 1992; Brown and Murphy-Walker 1996; Röll and von Düring 2008; Trifonov et al. 2015). However, these occasional males appear to be infertile, either lacking mature spermatozoa or possessing deformed spermatozoa (Yamashira and Ota 1998; Röll and von Düring 2008).

Parthenogenetic organisms are ideal laboratory animals for developmental studies because there is no need for mate-pairing, every individual is reproductively active, and individuals within clonal lineages are genetically identical. However, few parthenogenetic reptiles are routinely bred and maintained in laboratory settings (Cole and Townsend 1977; Darevsky et al. 1985; Maslin 1971; Kearney and Shine 2004; Lutes et al. 2011). *Lepidodactylus lugubris* has other desirable characteristics including high fecundity, ease of captive care, and fast maturation. Furthermore, this species is easily available via targeted field collection or through the pet trade, space-efficient to keep, and lays hard-shelled eggs (Fig. 1), making it tractable to perform embryological work. Herein we describe detailed methods for the laboratory maintenance of captive *L. lugubris* and embryo collection to serve as resources for researchers investigating developmental morphology, sexual development, parthenogenesis, and cytogenetics. These protocols will serve as a foundation for laboratory research on *L. lugubris* to accompany forthcoming genetic and embryological resources.

HUSBANDRY

Source of animals.—To establish a laboratory colony, we collected 20 wild adults from populations in the Hawaiian Islands (USA) in 2009 and 2012 under permit from State of Hawaii Division of Forestry and Wildlife (Permits: EX09-06, EX12-08). Clones A, B, and C are present in Hawaii (Fig. 2; Stejneger 1899; Cuellar 1984; Zug 2013); however, C clones are markedly rarer (Moritz et al. 1993). *Lepidodactylus lugubris* of either A clone or B clone varieties are also readily available in the U.S. and European pet trade (pers. obs.).

Housing, humidity, temperature, and cleaning.—We keep between 2–5 adults housed together in a single enclosure. Because this species is parthenogenetic, issues concerning sex ratio are nonexistent, and thus, any combination of individuals of roughly the same size can be housed together. However, we suggest placing individuals of the same clone types together, which will allow for easier allocation of clone types for clone-specific research. Furthermore, we have observed that A clones may behave aggressively toward spotted clone B types resulting in weight loss in the B clones and the need to keep those clones separately. *Lepidodactylus lugubris* can exhibit intraspecific aggression comparable to behaviors observed in other small gecko species (e.g., *Hemidactylus frenatus*; Brown and Sakai 1988; Brown et al. 1991; Petren et al. 1993) although they do not appear to engage in the near-lethal battles of some sexual gecko species (e.g., *Eublepharis macularius*; Mason and Gutzke 1990; Brillet 1993). Despite this, aggression between captive *L. lugubris* can occur, and we suggest immediately isolating any geckos that exhibit bite marks or excessive weight loss due to intraspecific aggression or reproductive stresses. Brown and Sakai (1988) demonstrated that isolated gravid individuals exhibit lower fecundity than those in social groups. We therefore recommend against keeping individuals in isolated enclosures if the aim is to maximize egg production. Brown and O'Brien (1993) provide evidence that keeping two individuals per enclosure allows for the highest reproductive output because subordinates in *L. lugubris* dominance hierarchies typically take longer to reach sexual maturity.

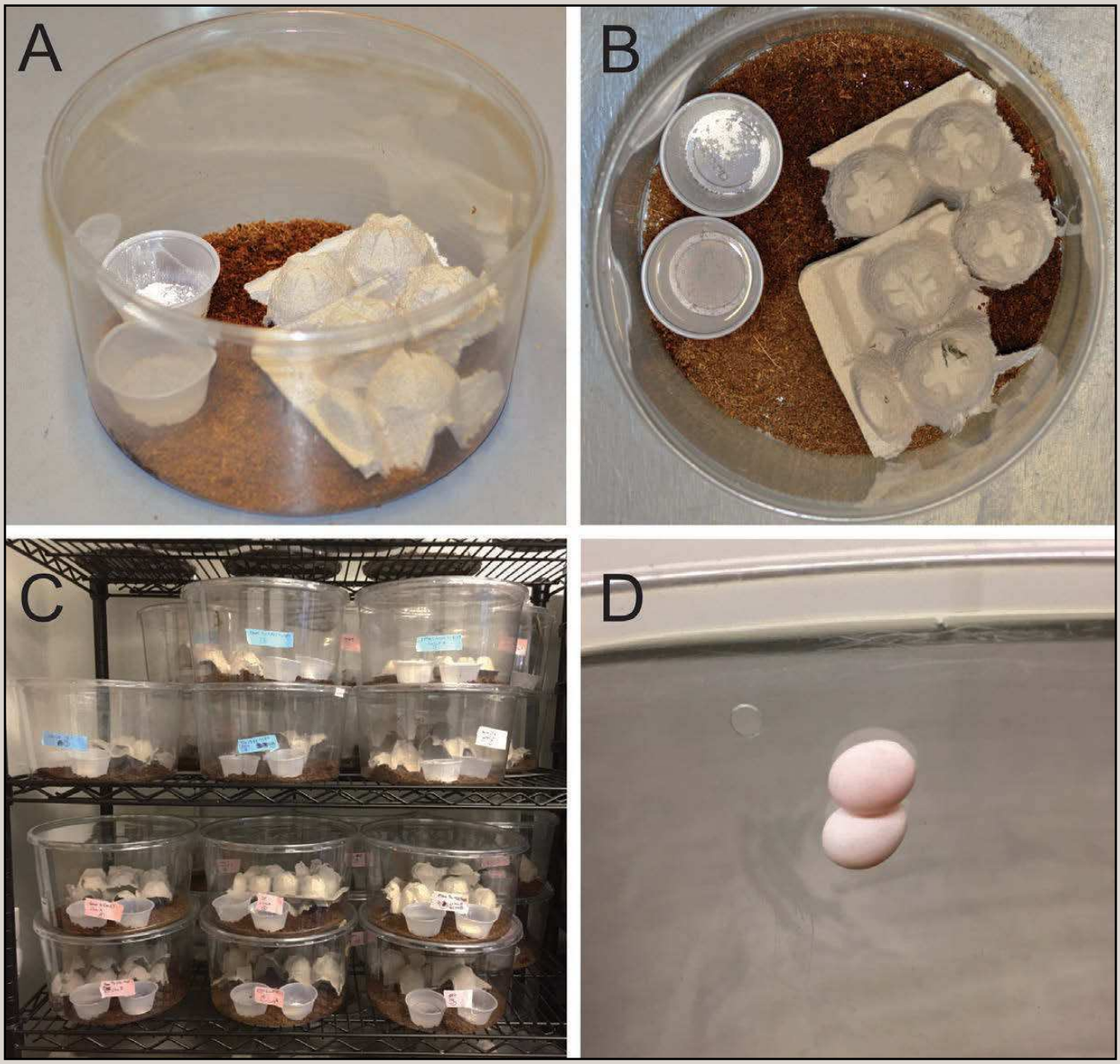

FIG. 3. A) A typical enclosure for captive *Lepidodactylus lugubris* showcasing coconut fiber substrate, cardboard egg carton shelter, a water dish, and a dish of calcium supplement. B) The same enclosure from an aerial view. C) A space efficient collection of 23 *L. lugubris* enclosures collectively housing 68 individuals. D) A clutch of *L. lugubris* eggs adhered to the wall on the enclosure.

Geckos can be kept in a variety of cage designs including glass aquaria with screen lids or plastic cages with ventilated tops. We chose to use oversized ventilated deli cups as they are space efficient and maintain relatively high humidity. Maintaining high humidity (~50–80%) is important when ambient humidity is low, such as arid regions or in winter in temperate regions. Each enclosure consists of an oversized plastic deli container (9-7/8 inches × 5-1/2 inches; 25.08 cm × 13.97 cm; purchased from www.superiorshippingsupplies.com) with 12 4-mm diameter holes in the side and a 10-inch matching plastic lid (Fig. 3A, B). These enclosures easily can be kept on a wire or stainless-steel shelf (Fig. 3C). We use approximately 15 mm of loose coconut fiber (Exo Terra®) as a substrate. After rehydrating coconut fiber from its typical commercial "brick" form, we allow coconut fiber to dry out until no water drips out when squeezed firmly by hand. This typically provides the ideal amount of substrate moisture and cage humidity. Fragments of pulp fiber egg cartons can be stacked on top of each other to provide hiding spots and shelter (Fig. 3A, B). Cages are cleaned every two weeks. We transfer geckos to a clean cage and then empty out the dirty enclosure and wash/disinfect the old cages in bulk. The washing process proceeds as follows: scrub with dish soap and water, rinse with clean water, soak in a 5% bleach solution for five minutes, and rinse with water again prior to air-drying.

Enclosure temperature and humidity for both postnatal individuals and eggs should range between 24.0–28.0°C and 30–40%, respectively. If needed, an additional heat source, such as a heat pad (FlexWatt Heat Tape or Ultratherm Heat pad), can be

TABLE 1. Preservation and associated storage methods for *Lepidodactylus lugubris* embryos for assorted laboratory techniques. As different or additional methods for DNA or RNA storage may be required depending on the reason for the tissue being collected, refer to Gamble (2014). EtOH, ethanol; MeOH, methanol; PFA, Paraformaldehyde; RT, room temperature (23°C).

Technique	Preservation Method	Storage Method
Specimen preparation	Fix overnight in 10% formalin	Dehydrate to 70% EtOH, store at RT
Immunohistochemistry	Fix 2 hours in 4% PFA at 4°C	Dehydrate to 100% MeOH, store at -20°C
Electron microscopy	1% glutaraldehyde at 4°C	1% glutaraldehyde, store at 4°C
Histology	Fix overnight in 10% formalin	Dehydrate to 70% EtOH, store at RT
In situ hybridization	Fix overnight in 4% PFA at 4°C	Dehydrate to 100% MeOH, store at -20°C
DNA extraction	95% EtOH	95% EtOH, store at RT
RNA extraction	Trizol or snap freeze in liquid nitrogen	Store at -80°C

placed underneath approximately one third of the container to ensure a temperature gradient is established. The additional heat source should not be placed underneath the water dish, which would result in superfluous humidity within the enclosure. If the enclosure becomes too humid, wipe condensation off of the sides of the enclosure as needed. Furthermore, the heat source should never cover more than ~30% of the cage bottom to allow natural thermoregulation within the cage and avoid over-heating. Different clone types vary in their temperature preferences (Bolger and Case 1994) with spotted B clones preferring lower temperatures than A and other B clones. *Lepidodactylus lugubris* in both captive and natural settings are nocturnal but occasionally forage during the day (Oliver and Shaw 1953; Perry and Ritter 1999). We expose our colony of *L. lugubris* to 14 h of ambient light, from 32-watt fluorescent ceiling light fixtures, each day. This species, like other nocturnal gecko species, does not require special UV-A or UV-B lighting (de Vosjoli et al. 1998).

Food and water.—Water is available *ad libitum* in 2-oz plastic cups (aka soufflé cups – SOLO®) and changed weekly or more frequently if necessary. Geckos are fed two to three times/week. Because *L. lugubris* are both insectivorous and nectivorous (Perry and Ritter 1999), we alternate diets every feeding. A typical feeding of insects consists of approximately 7–10 small insects per gecko, either two-week-old crickets (*Gryllodes sigillatus* or *Acheta domestica*) or small mealworms (*Tenebrio molitor*). A typical feeding of a fruit-based prepared diet consists of approximately 0.5 ml per individual gecko of liquid diet such as Repashy Crested Gecko Meal Replacement (Repashy Ventures Inc.) or Pangea Gecko Diet Breeding Formula (Pangea Reptile LLC). Prepared gecko diets are offered in small SOLO soufflé cups and any uneaten food should be removed from the enclosure the following day. *Lepidodactylus lugubris* use large amounts of calcium when egg-laying so a small cup of powdered calcium supplement with vitamin D3, e.g., SuperCal HyD (Repashy Ventures Inc.), should be made available at all times to prevent metabolic bone disease (de Vosjoli et al. 1998). *Lepidodactylus lugubris*, indeed most gecko species, will eat the calcium directly out of the cup (deVosjoli et al. 1998; pers. obs.).

Oviposition, egg collection, and juvenile care.—*Lepidodactylus lugubris*, like other gekkonids, lay hard-shelled eggs with a fixed clutch size of two eggs via synchronous single ovulation from each ovary (Kluge 1967; Bustard 1968; Jones et al. 1978; Fig. 3D). *Lepidodactylus lugubris* occasionally oviposit a single egg (Sabath 1981). Although no empirical data exist for ovarian cycle length for *L. lugubris*, egg-laying occurs year-round (Oliver and Shaw 1953; Jones et al. 1978). Individuals adhere or "glue" eggs to a variety of surfaces (Oliver and Shaw 1953). We most often find eggs adhered to the walls of the enclosures (Fig. 3D) and underneath pulp fiber egg cartons. If cork bark fragments are available, geckos preferentially lay eggs in the cracks in the bark. *Lepidodactylus lugubris* occasionally eat their own eggs (Miller 1979); therefore, we separate eggs from the adults frequently and in tandem with cage cleaning. Upon cleaning, we set any enclosures with eggs aside, with coconut fiber remaining to provide humidity. Egg enclosures remain at the same temperature as adult and juvenile enclosures. Besides being checked every day for hatched juveniles, egg enclosures can be left alone until hatching or embryo collection. Incubation time post-oviposition exhibits an inverse linear relationship with incubation temperature (Brown and Duffy 1992). Brown and Duffy (1992) demonstrated that eggs from a single *L. lugubris* can vary in incubation times, extremes being between approximately 65 days post-oviposition (dpo) at an average temperature of 25.5°C and 103 dpo at 22.0°C. Upon hatching, juveniles are approximately 16–21 mm SVL and can be housed with clutch-mates. Housing, humidity, temperature, feeding schedule, and cleaning schedule of juveniles are consistent with those of adults although live food items are smaller, e.g., pinhead crickets, newly hatched mealworms, and flightless fruit flies (*Drosophila melanogaster* or *D. hydei*).

Euthanasia.—Although we are keeping and breeding *L. lugubris* for embryo production it is necessary to have a protocol available for euthanasia in the event of an emergency, i.e., a lizard has been injured or is in severe distress. Lizards, both postnatal and near full-term embryos, can be euthanized by injection with tricaine methanesulfonate (MS-222), an approved method for reptile euthanasia in the 2013 AVMA Guidelines on Euthanasia (Leary et al. 2013), using the two-stage procedure from Conroy et al. (2009).

Embryo Collection, Preservation, and Storage

The eggs of *L. lugubris* are often firmly adhered to the side of the cage or on the underside of items within the cage. Eggs that are not fully adhered to a surface by the female often desiccate several days after laying. Regardless of how the egg is positioned by the female, the embryo rotates to the uppermost surface of the eggshell and can easily be visualized by shining a light through the shell from the backside (i.e., "candling"). The eggs cannot be easily removed from their surface by hand without cracking the shell and damaging the embryo inside. To remove an intact egg, we use a #11 scalpel to cut the egg off of the surface it is adhered to. The egg can then be handled by hand or by using a perforated spoon.

To dissect the embryo from its shell, we first submerge the egg in 1X phosphate buffered saline (PBS) in a glass culture dish. Once submerged the eggshell can be gently cracked and the pieces removed using #5 watchmaker's forceps. This leaves an intact bolus of yolk and embryo surrounded by translucent membranes. Using a pair of #5 forceps we free the embryo from the yolk and membranes. The embryos can now be easily moved, once again using a perforated spoon, to a clean dish of DEPC-treated (i.e., RNAase free) PBS. To separate the primary nutrient stalk from the embryo without damaging the viscera, we create a clean cut by pinching the stalk with one set of forceps and then sliding the second set along its length, crossing the stalk.

The method of embryo preservation and storage ultimately depends on the long-term use of the specimen. In our labs, we use *L. lugubris* embryos for morphological analysis, nucleic acid extraction, histology, scanning electron microscopy, immunohistochemistry, and *in situ* hybridization of mRNA localization. We have summarized the preservation and storage techniques used for *L. lugubris* embryos in Table 1.

Compared to working with adult specimens, there are several critical differences to processing embryonic material. First, moving between aqueous and alcohol-based solutions needs to occur gradually. Rapid changes in tonicity, such as when moving between PBS and alcohol, will lead to dramatic wrinkling of the outer epithelium and may deform the embryonic morphology. We suggest a three step gradual transition between aqueous and alcohol-based solutions (e.g., 25%, 50%, 70% ethanol). In addition, fixing tissue at 4°C helps to reduce the chances of tissue degradation during fixation. Finally, because of the small size of the embryos and variable levels of gene expression, all solutions should be maintained in sterile or RNAase-free conditions.

Conclusions

Using the protocols described in this paper, we have successfully maintained a colony of *Lepidodactylus lugubris* in a space-efficient and inexpensive manner. Furthermore, our collection of nearly 60 individuals is capable of producing approximately 50 eggs per month, making *L. lugubris* an ideal amniote for embryological research. These protocols were created with a basic knowledge of gecko captive care and natural history, and, with additional optimization, can be applied to other similar gekkonid species. *Lepidodactylus lugubris* is an ideal model to study vertebrate evolutionary developmental biology and parthenogenesis in a laboratory setting. With these protocols, we hope to set a foundation to make this emerging model species more accessible.

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