Abstract

HULL, KAITLYN CHRISTINE. Restriction Enzyme Mediated Integration and Lentiviral Transgenesis of the Species *Eublepharis macularius*, *Litoria caerulea*, and *Xenopus laevis*. (Under the direction of Dr. Paul Mozdziak.)

Restriction enzyme mediated integration (REMI) transgenesis and lentiviral transgenesis are effective methods of introducing transgenes into the genome of model organisms. Two species of frog and one species of gecko were chosen for transgenesis. REMI was attempted on the two frog species, white’s tree frog *Litoria caerulea*, and the African clawed frog *Xenopus laevis*. Hatchling *Xenopus* tissue samples were collected and observed for fluorescence under brightfield and fluorescent light. REMI was successful in producing transgenic full term *Xenopus* embryos but unsuccessful in producing development past neurulation in *Litoria*. Further methods of transgenesis were attempted on *Litoria caerulea*. Two methods of lentiviral transgenesis were developed and applied to *Litoria caerulea* and the leopard gecko *Eublepharis macularius*. The lentiviral methods produced required two protocols for the different species; hatchling *Litoria* tadpoles were incubated overnight in a lentiviral pseudoparticle bath, and newly laid *Eublepharis* eggs were directly injected with lentiviral pseudoparticle media for infection. Histological evaluation revealed the presence of DsRed in lentiviral infected gecko and frog organs with protein production concentrated in the muscle, kidney, heart, and brain. Of the injected gecko eggs, 89% of efficiency of transgenesis was confirmed using PCR screening.
Restriction Enzyme Mediated Integration and Lentiviral Transgenesis of the Species, 
_Eublepharis macularius_, _Litoria caerulea_, and _Xenopus laevis_

by
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Transgenesis, the integration of foreign DNA into a eukaryotic host genome, has been attempted in many species including fish, amphibians, birds, and mammals (Gama Sosa et al., 2009; Gordon et al., 1980; Macha et al., 1997; Mozdziak and Petitte, 2004; Zelenin et al., 1991). Transgenic animals have been produced for studies of gene function, organogenesis, and aging (Gama Sosa et al., 2009; Yergeau et al., 2010). Techniques most commonly used for gene transfer are DNA microinjection, transposons, Restriction Enzyme Mediated Integration (REMI), and retroviral vectors (Houdebine, 2002). All techniques involve transduction of foreign DNA into a host genome, but the methods differ in delivery, transgene expression, and production efficiency.

DNA pronuclear microinjection is one of the earliest forms of transgenesis. In this technique, foreign DNA is injected into a recently fertilized egg where the gene is then randomly incorporated into the embryo's genome (Sible and Wroble, 2008). A disadvantage to using DNA microinjection is that expression varies, typically producing mosaic expression with a probability of non-integration (Andres et al., 1984; Bendig and Williams, 1984; Chesneau et al., 2008; Etkin et al., 1984; Etkin and Roberts, 1983; Rusconi and Schaffner, 1981). Transgene integration frequency is low when using microinjection; in studies involving zebrafish and medaka the frequency of integration ranged between 1-10% (Grabher and Wittbrodt, 2009; Stuart et al., 1988; Stuart et al., 1990; Tanaka and Kinoshita, 2001).
Transposon transgenesis is a method similar to pronuclear microinjection. The foreign DNA is ligated into the transposon DNA which is then injected with synthetic transposase mRNA into a fertilized egg to cause random integration into the host genome (Yergeau et al., 2010). Mosaic expression is common due to the process requiring co-injection of transposase mRNA to catalyze the insertion reaction; if the transposase mRNA is not transcribed before the embryo cells complete the early stages, the transposase enzyme will not be available to catalyze the insertion reaction in those cells (Yergeau et al., 2010). Even so, recent studies in medaka and zebrafish using a Sleeping Beauty transposon system have shown a transgene production efficiency reaching 30% (Grabher and Wittbrodt, 2009). A disadvantage to using transposon transgenesis is that its efficiency is inversely related to the inserted transposon's size making it an inefficient method for large gene insertion that is typically required for transgenesis (Grabher and Wittbrodt, 2009). Transposons also require species-specific cofactors limiting its application to thoroughly studied species (Grabher and Wittbrodt, 2009).

Restriction enzyme mediated integration (REMI) is a method of transgenesis found to be efficient for producing transgenic *Xenopus laevis* (Gama Sosa et al., 2009). In this method, restriction enzyme (RE) modified sperm nuclei are incubated with RE modified foreign DNA and injected into an unfertilized egg, which produces non-mosaic integration. Transgenesis efficiency was greater than that of microinjection with transduction ranging between 10% and 50% (Amaya and Kroll, 2010). A disadvantage of REMI is that success is dependent on technical skills and the availability of high quality eggs and sperm.
Retroviral vectors are widely used in studies of molecular and cell biology, biomedicine, and biotechnology. The gene of interest is inserted into a replication defective retroviral vector, which is then transduced with helper plasmids into producer cells to create replication defective retroviral particles (Saenz and Poeschla, 2004; Spirin et al., 2008). Transgenesis is achieved by infecting the host with modified retroviral particles, which transduce the viral DNA with the gene of interest into the host's genome. An advantage of a branch of retroviruses, the lentivirus, is that they can transduce both dividing and non-dividing cells leading to high infection rates (Spirin et al., 2008). Lentivirus vector transgenesis has higher transduction efficiencies with higher expression than microinjection (Pfeifer, 2004; Pfeifer and Ikawa, 2002; Spirin et al., 2008). In studies involving transgenesis of pig embryos, a high level of expression was observed in 65% of the subjects (Spirin et al., 2008). A limitation to lentiviral transgenesis is the gene cannot exceed 8kb in size, whereas in microinjection there is no size limit (Spirin et al., 2008).

In the following studies, transgenesis was attempted in two frog species, *Xenopus laevis* and *Litoria caerulea*, and one gecko species, *Eublepharis macularius*. The REMI technique was applied to the two frog species because of its previous success in producing transgenic *Xenopus laevis* as well as its advantages over microinjection and transposon methods. Novel techniques of lentivirus infection were developed and applied to *Litoria caerulea* and *Eublepharis macularius*. 
**REMI Transgenesis**

Restriction enzyme mediated integration (REMI) transgenesis is a large-scale method used to incorporate foreign DNA into a subject’s genome. REMI can be performed without expensive equipment or a large time dedication (Haeri and Knox, 2012). REMI success, however, is dependent on the availability of high quality eggs, sperm nuclei, egg extract, and purity of the linearized DNA.

REMI requires 5 steps: preparation of oocyte extract; preparation of sperm nuclei; restriction enzyme-mediated integration; microinjection of digested sperm and plasmids into unfertilized eggs; and selection of transgenic embryos. Oocyte extract is needed to activate the sperm, de-condensing the sperm chromatin for integration of the gene of interest into the sperm genome (Kroll and Kirschner, 1999). Sperm nuclei preparation involves de-tailing the harvested sperm and suspending it in a dilution buffer for storage. Before use, the sperm nuclei are digested with restriction enzymes to allow insertion of the DNA of choice. The DNA is linearized prior to use with the same restriction enzymes to ensure cohesive ends with the sperm DNA. Integration of the foreign DNA into the sperm is random (Kroll and Kirschner, 1999). Unfertilized eggs are injected with the sperm nuclei and plasmids after treatment with a de-jelly solution which increases ease of injection. After injection of the sperm, integration into the embryo’s genome occurs as the digested DNA is repaired during the initial developmental stages. The level of expression in transgenic animals produced by REMI varies between individuals, as integration events are independent. Because the sperm is digested, genetic abnormalities are high. On average, 1/3 of injected embryos are expected
to receive a single nucleus and develop properly (Chesneau et al., 2008). A single insertion event is typical of early REMI experiments and produces non-mosaic animals, but when stored sperm nuclei are used, chromosomal breaks can form. These can be the cause of multiple integration sites in the subject’s genome and cause mosaic expression and malformations (Bronchain et al., 1999; Kroll and Kirschner, 1999; Marsh-Armstrong et al., 1999). Mosaic distribution of transgene can also be caused by the transgene being maintained as extrachromosomal structures (Etkin and Pearman, 1987)

Fig. 1: REMI transgenesis flow chart. Sperm nuclei and the vector of interest are restriction enzyme digested and incubated together to allow integration. The sperm nuclei solution is then injected into an unfertilized egg and transgenic embryos are selected.
Retroviral transgenesis is the most widely used method of transgene delivery in applied biological fields (Spirin et al., 2008). A problem with retroviral transgenesis is that retroviruses cannot infect dividing cells because the provirus and viral proteins cannot enter the intact cell’s nucleus. This problem does not occur when the cells are infected with a branch of complex retroviruses i.e., the lentivirus. Lentiviral proteins pass through the nuclear pores to integrate the DNA into the cell’s genome (Spirin et al., 2008). Feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV40), and equine infectious anemia virus (EIAV) are common lentiviral vectors used in transgenesis. FIV, HIV, and SIV have a central DNA flap region, which is presumed to help enable the lentivirus to enter the nucleus (Zennou et al., 2000).

Lentiviral vectors were first developed from the human immunodeficiency virus (HIV) (Naldini et al., 1996; Poeschla et al., 1998). Non-primate lentiviral vectors were developed during the post-AIDs era because of HIV’s pathogenic mechanism; they do not cause human disease while still being efficient for transgene inoculation (Saenz and Poeschla, 2004).

The first FIV vector was constructed from FIV 34TF10 (Saenz and Poeschla, 2004). FIV vectors have since been reengineered so packaging and transfer vector functions, as well as the general efficiency of the lentivirus transduction have improved. Lentiviruses have sequences coding for Gag, Pol and Env proteins (Zennou et al., 2000). The FIV genome is simpler than the SIV, HIV, and EIAV genomes. FIV vectors contain accessory genes for
three non-structural proteins (vif, ORFA/ORF2, rev), where the later viruses have regions encoding six non-structural proteins (Saenz and Poeschla, 2004). Lentivirus vectors have been tailored so the genes known to be important for pathogenesis were deleted (Desrosiers et al., 2001). For example, third generation lentiviruses are missing the RRE sequence and accessory genes that are required for transduction efficiency (Kim and Mitrophanous, 1998). FIV has been used to transduce non-dividing and dividing cells of the brain, eye, airway, hematopoietic system, liver, muscle, and pancreas (Curran et al., 2000; Johnston et al., 1999; Poeschla et al., 1998; Song et al., 2003; Wang et al., 1999).

Lentivirus transgenesis begins with the mature virus binding to a receptor on the cell surface. The lentivirus and the cell membrane fuse exposing the viral RNA and accessory proteins to the cytoplasm of the cell. The RNA is reverse transcribed and provirus DNA is produced. The DNA, viral and cell proteins form a pre-integration complex (PIC) and enter the cell’s nucleus through nuclear pores via a complicated mechanism. The provirus is integrated into the cell’s genome via nonhomologous end joining. The host cell later transcribes the integrated DNA with its genomic DNA (Spirin et al., 2008). In this manner, lentiviral transgenesis has been used to produce transgenic animals including mammals (mouse, rat, pig, bovine), avian, fish and various amphibians (Nakagawa and Hoogenraad, 2011; Pfeifer and Hofmann, 2009).
**Lentiviral Vector pCDF1**

Vector pCDF1 is a third generation FIV lentiviral vector. Because 293T cells are used to package the virus pseudoparticles, the human cytomegalovirus (hCMV) enhancer/promoter is encoded in the lentivirus vector (Zennou et al., 2000). The hCMV sequence in the pCDF1 FIV vector is imbedded in the chicken β-actin promoter forming the CAG promoter. The CMV/chicken beta-actin promoter drives transgene protein production in the liver, brain, heart, kidney, spleen and lung tissues (Fahim et al., 2009).

**pCDF1-MCS1 Vector**

![Diagram of pCDF1-MCS1 Vector]

- Cytomegalovirus/5’Long Terminal Repeat Promoter: 1-415 bp
- gag Packaging Signal: 762-1011 bp
- RRE Rev Response Element: 1012-1143 bp
- cPPT Central Polyuridine Tract: 1150-1391 bp
- Cytomegalovirus Promoter: 1407-1746 bp
- Multiple Cloning Site 1: 1747-1816 bp
- Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element: 1817-2406 bp
- 3’Long Terminal Repeat Promoter: 2525-2740 bp
- SV40 Poly-A Termination Signal: 2741-2872 bp
- SV40 Origin: 2881-3027 bp
- pUC Origin: 3397-4070 bp
- Ampicillin Resistance Gene: 4215-5075 bp

Fig. 2: pCDF1-MCS2 lentiviral backbone vector construct. Construct includes CMV promoter, sequences for the proteins gag, RRE, cPPT, multiple cloning site (MCS1), woodchuck hepatitis virus posttranscriptional regulatory element, 3’long terminal repeat promoter (LTR), SV40 Poly-A termination signal, SV40 origin, pUC origin, and ampicillin resistance gene.
The pCDF vectors contain the following: CMV promoter; Multiple Cloning Site (MCS); WPRE element; SV40 polyadenylation signal; hybrid CMV-5LTR promoter; genetic elements (cPPT, GAG, LTRs); SV40 origin; pUC origin; and Ampicillin resistance gene (Figure 2).

Fig. 3: pCDF1 lentiviral transfection flow chart. The packaging plasmids and expression plasmid are co-transfected into 293TN Producer Cells. Viral supernatant is then collected and applied to NIH 3T3 cells for titration.
For lentiviral pseudoparticle generation of third generation lentiviruses, three vectors are needed; two packaging plasmids (pPACKF1 Packaging Plasmid mix, SBI); and an expression vector (pCDF1-MCS1 cDNA expression vector) for gene delivery (SBI, Mountain View, Ca). The packaging vectors contain sequences needed for packaging and transcription of the expression vector. The vectors are transfected into 293T cells, and pseudoviral particles are collected (Figure 3). To titer, the viral particles are applied to NIH 3T3 cells and assayed.

DsRed Protein

Fluorescent proteins (FPs) occur naturally in organisms of four phyla (Cnidaria, Ctenophora, Arthropoda, and Chordata) including jellyfish, crustaceans, comb jellies, and the cephalochordate amphioxus (Chudakov et al., 2009; Deheyn et al., 2007; Haddock and Case, 1999; Shagin et al., 2004). It has been hypothesized that FPs are used in their indicative organisms as a photosynthesis modulation and for optical communication (Chudakov et al., 2009). FPs are tools used in biotechnology to visualize cell attributes such as morphology, location, movement, and mitotic stages (Chudakov et al., 2009). Wild type GFP protein was discovered in 1962 by Osamu Shimomura in the species Aequorea victoria and was later cloned and sequenced in 1992 by Douglas Prasher (Prasher et al., 1992). GFP was first introduced to cell culture in 1994 (Zhu and Zon, 2004). GFP has been used for tracking gene expression, whole-cell labeling, labeling organelles, and protein labels (Chudakov et al., 2009; Strack et al., 2010; Zhu and Zon, 2004). GFP transgenic organisms include the worm Caenorhabditis elegans, the fish Danio rerio and Oryzias latipes, rodents Mus muscles and
*Rattus norvegicus*, fly *Drosophila melanogaster*, slime mold *Dictyostelium*, plants *Arabidopsis thaliana* and maize, and the amphibians *Xenopus laevis* and *tropicalis* (Bronchain et al., 1999; Chalfie et al., 1994; Hamada et al., 1998; Hirsch et al., 2002; Hodgkinson, 1995; Hu and Cheng, 1995; Ikawa et al., 1995; Marsh-Armstrong et al., 1999; Peters et al., 1995; Sheen et al., 1995; Takeuchi et al., 2003; Wang and Hazelrigg, 1994).

Engineering via point mutations have made spectral variants of GFP possible, barring the red spectrum (Schmid and Neumeier, 2005). DsRed was discovered in an effort to find a red fluorescent tag. A fluorescent homologue of GFP isolated from *Discosoma* coral, DsRed has the longest wild type emission wavelength of 583 nm (Baird et al., 2000; Zhu and Zon, 2004). DsRed is an oligomeric fluorescent label that has been used extensively in research involving microscopy and flow cytometry (Strack et al., 2010). The DsRed protein forms a self-aggregating tetrameric structure where each monomer contacts two other monomers. This presented problems in DsRed’s development, inhibiting its use as a genetic fusion tag (Zhu and Zon, 2004). However, DsRed has a high extinction coefficient and a resistance to pH extremes that make it a viable candidate for transgenic research. DsRed and the GFP homologues have been used in recent studies to produce transgenic animals including mammals (mouse, rat, pig, bovine), avian, fish and various amphibians (Nakagawa and Hoogenraad, 2011; Pfeifer and Hofmann, 2009).
*Xenopus laevis, Litoria caerulea*  
*and Eublepharis macularius*

When *Xenopus* were first introduced as laboratory animals they were subjects for pregnancy diagnosis (Shapiro and Zwarenstein, 1933). Use of *Xenopus* expanded to include pharmacological tolerance and growth regulation studies (Grimm, 1951). Today *Xenopus Laevis* is used in embryology, genetics, and molecular studies. The availability of the *Xenopus* genome, their general robustness, ease of care, and ability to reproduce year round when induced with hormones make *Xenopus* a viable subject for study (Chesneau et al., 2008; Kroll and Kirschner, 1999). Most recently *Xenopus* have been introduced in to the field of transgenesis for embryology, misexpression, and transcriptional regulation studies (Kroll and Kirschner, 1999).

*Litoria caerulea* (White’s tree frog) is a common tree frog found in Australia. Frequently found inhabiting human dwellings, *Litoria* are considered docile and have been bred in the pet trade. *Litoria* have been used in previous studies as subjects to study parotoid gland secretion, natural insect repellents, lungworm infections, wet adhesion, skin water loss properties, and hind limb locomotion studies (Christian and Parry, 1997; Crockett and Peters, 2007; Pizzatto and Shine, 2006; Scholz et al., 2008; Smith et al., 2003; Williams et al., 2006).

Leopard geckos are produced in large numbers due to their hardiness and tractability (De Vosjoli et al., 2005; Seufer et al., 2005; Thorogood and Whimster, 1979; Wise, 1997; Wise et al., 2009; Wagner, 1974). *Eublepharis macularius*, the leopard gecko, is a medium sized
gecko indigenous to Afghanistan, northwestern India, and Pakistan. Adult leopard geckos typically weigh 45-60 grams and reach typical lengths of 20 centimeters. Leopard geckos are one of the most easily cared for lizards, which makes them one of the most widely bred reptiles (De Vosjoli et al., 2005). Because of the leopard gecko’s popularity, captive bred lines are available negating the need to obtain wild caught individuals (Wise et al., 2009). Captive bred lines of leopard geckos also demonstrate a range of morphological differences including pattern variations such as jungle, striped, patternless, and color variations such as tangerine, white, lavender, melanistic, amelanistic, and leucistic (De Vosjoli et al., 2005). In previous studies, leopard geckos have been used as models for reproductive physiology, central nervous system development, tissue regeneration, and studies of gene expression (Bull, 1987; Valleley et al., 2001; Whimster, 1978). Leopard geckos were chosen for method of transgenesis because of their general hardiness, captive bred genetic lines, and because their eggs were soft shelled and large, measuring 25 x 12 mm on average. This assisted in determining location of gecko embryos (Minton, 1966).


Restriction Enzyme Mediated Integration and FIV Lentiviral Transgenesis Applied to Amphibians

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A REMI and FIV Transgenesis Applied to Amphibians

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ABSTRACT: Restriction enzyme mediated integration (REMI) transgenesis and lentiviral transgenesis are effective methods of introducing transgenes into the genome of frogs. One aquatic amphibian species, *Xenopus laevis*, and one land dwelling species, *Litoria caerulea*, were chosen as subjects for transgenesis. REMI produced *Xenopus laevis* that expressed the fluorescent protein DsRed. REMI was unsuccessful in producing transgenic *Litoria* therefore lentiviral transgenesis was attempted. Hatchling *Litoria* tadpoles were incubated overnight in replication defective lentivirus particles containing the coding sequence for DsRed. Histological evaluation revealed the presence of DsRed in brain, heart, liver, kidney, and muscle tissues. Therefore, lentiviral transgenesis appears to be a viable technique for producing transgenic land-dwelling frogs.

[Key words: DsRed, *Xenopus laevis, Litoria caerulea*, REMI, Lentivirus]
INTRODUCTION

In recent years the African Clawed Frog *Xenopus laevis* has become a leading model organism for transgenesis. Restriction enzyme mediated integration (REMI) of DNA into sperm nuclei, and transplantation into unfertilized eggs, is a method that has been used previously to produce GFP transgenic lines of *Xenopus laevis* (Sakamaki et al., 2004). Although positive results have been produced using the *Xenopus* cardiac actin promoter, low expression in tissues has been a problem (Sakamaki et al., 2004). The CAG promoter is a combination of the cytomegalovirus early enhancer element and the chicken beta-actin promoter; it drives higher expression than the *Xenopus* cardiac actin promoter (Sakamaki et al., 2004). The CAG promoter has driven transgenic expression in most *Xenopus* tissues, including germline cells (Marsh-Armstrong et al., 1999). Transgenic *Xenopus* progeny show germline transmission without the CAG promoter, but expression is mosaic (Marsh-Armstrong et al., 1999). DsRed is a 28-kDa red homologue of the fluorescent protein GFP, isolated from the *Discosoma* species of coral (Matz et al., 1999). The development of DsRed transgenic *Xenopus* would support the use of *Xenopus* as a model organism because DsRed emits in the visible light spectrum and would assist in visualizing developmental studies.

An objective of this study was to generate transgenic DsRed *Xenopus* as a potential model system for future biolabel and developmental studies. Although there have been several lines of transgenic *Xenopus* produced, no lines of transgenic land dwelling frogs exist. The main objective was to develop methods to generate transgenic land dwelling frogs. *Litoria*
caerulea (White’s tree frogs) is a common tree frog found in Australia. Frequently found inhabiting human dwellings, Litoria are considered docile and have been bred in the pet trade. Litoria have been used in previous studies as subjects to study parotoid gland secretion, natural insect repellents, lungworm infections, wet adhesion, skin water loss properties, and hind limb locomotion studies (Christian and Parry, 1997; Crocket and Peters, 2007; Pizzatto and Shine, 2006; Scholz et al., 2008; Smith et al., 2003; Williams et al., 2006). Litoria have the ability to produce several hundred to a thousand eggs in a single laying event.

MATERIALS AND METHODS

FROG HUSBANDRY METHODS

Adult Litoria caerulea Husbandry.

Litoria were maintained in sexed trios in 20-gallon aquariums at a room temperature of 27°C. Cage contents included a water bowl, drift wood branches, plastic foliage, with paper towel substrate. A one liter water bowl and was filled with 0.5L sterile water. Frogs were on a 12/12 fluorescent lighting schedule. Feeding took place two days a week and consisted of four to six crickets per frog dusted with Rep-Cal Herptivite and Rep-Cal calcium with D3 (Rep-Cal, Los Gatos, CA). Water bowl and water were changed once a week when cage washing and autoclaving took place. Before sterilization, bowls and cages were sprayed with a 5% bleach solution.
*Litoria* were placed in a rain chamber to induce breeding behavior. The rain chamber consisted of a 20-gallon aquarium with river pebble substrate, drift wood branches and plastic foliage, a platform to provide a dry area, and a Zoo Med (Costa Mesa, California) power sweep power head water pump with an outlet leading above the enclosure to a container that allowed the water to drip down into the aquarium to simulate rain.

**Adult *Xenopus laevis* Husbandry.**

*Xenopus* were maintained in sexed trios in two to five gallons of chlorine and chloramine free water at a room temperature of 21°C. Cage contents included PVC pipe and plastic plants for enrichment. Water was treated with 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts to bring the hardness to 215 ppm (Seachem Laboratories Madison, GA). *Xenopus* were placed on a 12/12 fluorescent lighting schedule. *Xenopus* were fed daily with *Xenopus* Express frog food (Brooksville, FL). Water changing took place once a week.

**Tadpole *Litoria* and *Xenopus* Husbandry.**

*Litoria* tadpoles were kept in groups up to seven tadpoles per liter of water. Water was treated with 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts to bring the hardness to 215 ppm (Seachem Laboratories Madison, GA). Water was heated with Flex Watt Heat tape to 27°C. A Tetra Whisper aquarium bubbler was used to aerate tadpole containers (Blacksburg, VA). Tadpoles were placed on a 12/12 fluorescent lighting schedule. Tadpoles were fed daily with *Xenopus* Express Premium Tadpole Diet.
(Brooksville, FL). Full container cleaning took place every other day, with half of the water changed every day to remove old food. Cleaning involved rinsing the containers with water and spraying and rinsing 2 times with 70% ethanol. Bowls were sterilized by baking in a laboratory oven at 180°C overnight every week.

*Tadpole Xenopus laevis Husbandry.*

Tadpoles were first raised in an incubator with 0.1x MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgSO$_4$, 2 mM CaCl$_2$, 5 mM HEPES (pH 7.8), and 0.1 mM EDTA) + 50 µg/mL gentamycin as a buffer. When tadpoles reached stage 35 of growth, they were transferred to a larger container (Nieuwkoop and Faber, 1994). Tadpoles were kept in 350 mL H$_2$O and fed 2-4 drops of tadpole food daily (*Xenopus Express Brooksville, FL*). Water pH was between 7.0-8.5 and water hardness was brought to 200 ppm. Water hardness was achieved by adding Cichlid Lake Salts at 6.4 g/L, and Equilibrium Salts at 0.8 g/L with sterile water. A Tetra Whisper aquarium bubbler was used to aerate tadpole containers (Blacksburg, VA). Room temperature was 21-24°C.

*Hormone Injections.*

*Xenopus laevis* were primed with a primary intraperitoneal (IP) injection of 200 International Units of Pregnant Mare’s Serum Gonadotropin (PMSG) five to seven days before induction of egg laying. To induce laying, 500 IU of human Chorionic Gonadotropin (hCG) were administered 10-12 hours before procedures were scheduled to begin. As soon as frogs were injected with hCG, they were transported to the lab for observation and egg collection.
Initially the *Xenopus laevis* hormone doses of PMSG and hCG were applied to *Litoria* but *Xenopus* doses did not induce egg laying. *Litoria caerulea* were primed with a primary IP injection of 200-300 International Units of PMSG five to seven days before induction of egg laying. To induce egg laying, *Litoria* were then given two IP injections of 250 IU of human Chorionic Gonadotropin (hCG) with 2µg Alarelin (synthetic luteinizing hormone releasing hormone) 12 hours apart. The first hCG injection occurred 12 hours before the procedure was scheduled to begin.

**RESTRICTION ENZYME MEDIATED INTEGRATION**

*Oocyte Extract Preparation.*

Oocyte preps were performed following the protocol detailed (Kroll and Amaya 1996). *Xenopus laevis* and *Litoria caerulea* were killed via double pithing after immersion in a 0.1% tricaine solution for 20 minutes or until unresponsive to needle poke. Ovaries were collected and macerated in a salt solution of 87 mM NaCa, 2.5 mM KCl, 1 mM MgCl$_2$, 1 mM Na$_2$HPO$_4$, 5 mM Hepes, and 1% Penn-Strep pH 7.8 (OR2) with collagenase A to digest at 28°C. After washing in the salt solution, eggs were sorted in 15 mM Hepes and 50 µg/mL gentamycin (OR3), washed with OR2, and placed at 15°C overnight in OR3. Oocytes were then washed 4 times in 1X extract buffer salts (XB) and rinsed again 2 times in cytostatic factor extract buffer salts (CSF-XB) with protease inhibitors at a 1:1000 dilution. XB solution consisted of 1X XB salts, 50 mM sucrose, and 10 mM HEPES. CSF-XB solution consisted of 1X XB salts, 1 mM MgCl$_2$, 10 mM HEPES (pH 7.7), 50 mM sucrose, 5 mM
EGTA. Protease inhibitors were 10 mg/mL Leupeptin, 10 mg/mL Chymostatin, and 10 mg/mL Pepstatin A. Samples were centrifuged for one min at 1000 rpm, excess buffer was removed, and solution was re-spun at 2000 rpm for one min or until 3 layers formed; the cytosolic layer was removed and centrifuged. The center most portion was harvested and frozen in liquid N₂.

_Sperm Nuclei Preparation._
Males were injected with 250 IU of hCG for _Xenopus_ and _Litoria_ an hour before killing to increase sperm production. To isolate sperm nuclei, testes were harvested and placed into a petri dish and rinsed with 1X Marc’s modified ringers solution (1X MMR) and 1X nuclear preparation buffer (1X NPB). 1X MMR consisted of 0.1 M NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM HEPES (pH 7.8), and 0.1 mM EDTA. 1X NPB consisted of 250 mM sucrose, 15 mM HEPES, 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride, 1 mM Dithiothreitol, and 2 mM of Ethylenediaminetetraacetic acid. Testes were then minced in 1X NPB and filtered through cheesecloth. Filtrate was centrifuged in an SS-34 rotor at 6,500 rpm (2,000 g) for 15 min at 4°C. _Xenopus_ sperm, pellets were warmed to room temperature and 50 nL of 10 nm/mL lysolecithin was added. For _Litoria_, lysolecithin alone did not subsequently de-tail the sperm; therefore an additional step of homogenizing with 100 strokes in a dounce homogenizer was needed before the sperm nuclei pellet was resuspended in 1X NPB/protease inhibitors/0.3% BSA. Protease inhibitors used were 10 mg/mL Leupeptin, 10 mg/mL Chymostatin, and 10 mg/mL Pepstatin A. Samples were centrifuged, and suspended in cold sperm storage buffer and frozen in
liquid nitrogen in 20 µL aliquots. Sperm nuclei storage buffer consisted of glycerol, 0.3% BSA, in 1X NPB. Sperm samples were stored at -80°C. Nuclei quality was evaluated by mixing sperm nuclei with oocyte extract. Only nuclear preparation that exhibited visible sperm nuclei swelling were used for injection.

De-jelly and Post de-jelly Solutions for Transgenesis.

For Xenopus, a 15% sodium borate (Sb) buffer (10mM NaOH pH to 8.5 with H$_3$BO$_3$), 2.5% cysteine free base pH 8.5 solution was used to de-jelly the eggs (Kroll and Amaya, 1996). To remove de-jelly solution eggs were rinsed 3 times in a solution of 0.5% bovine serum albumin (BSA) in 1x MMR.

Xenopus de-jelly solution was originally applied to Litoria, but the Litoria eggs are more delicate than the Xenopus eggs they exhibited visual dehydration, and embryos did not develop. A final solution of 5% Cysteine free base solution in 0.1X MMR, pH 8.5 was determined as an appropriate de-jelly solution. To remove de-jelly solution eggs were rinsed 3 times with a solution of 1% BSA in 0.4X MMR.

Attempts at injecting non-dejellied eggs involved laying a 1000 µm mesh over the groups of eggs and injecting through the mesh using the same needle sizes and injection medias as with the de-jellied eggs.
**Injection Media and Post-Injection Medias.**

*Xenopus* eggs were injected in a solution of 6% Ficoll 400 and 0.5% BSA in 0.4X MMR. Following injection, *Xenopus* eggs were placed in a solution of 6% Ficoll 400 and gentamycin (50 µg/mL) in 0.1X MMR.

Due to the *Litoria* eggs not activating when the *Xenopus* injection solutions were used, the solutions were adjusted. *Litoria* eggs were injected in a solution of 20X MMR, Ficoll 400, and 10% BSA. Following injection *Litoria* eggs were placed in a solution of 0.4X MMR, 2% BSA medium with 50 µg/mL gentamycin.

**REMI Egg Injection Procedure.**

The pCAG DsRed vector was chosen for transgenesis (Cambridge MA). Restriction enzyme SpeI and ApaLI digests were performed on the pCAG vector. SpeI cut the plasmid once at 18 bp; ApaLI cut the plasmid twice, at 4,010 bp and at 5,255 bp. The linearized plasmid contained an intact promoter and DsRed gene after restriction enzyme digest. Restriction enzymes were inactivated by placement in 4°C overnight. Sperm nuclei solutions used in nuclear microinjection to produce DsRed transgenic *Xenopus* and *Litoria* consisted of: sperm nuclei [50 sperm nuclei/nL], ApaLI or SpeI digested p-CAG DsRed plasmid, 0.5 IU of ApaLI or SpeI, oocyte extract, sperm dilution buffer, CaCl₂ and MgCl₂. After de-jellying, eggs were rinsed 3 times with post de-jelly rinse and loaded into a petri dish of injection media. Oocyte extract and sperm mix were combined and loaded into a 100 µm beveled
needle. Eggs were injected with 4-5 nL of sperm nuclei solution at a rate of 50 µL/hr. Sperm solutions were used up to 30 minutes after thawing.

An unpaired student t-test was applied to the population data; no significant difference was found between the SpeI transgenic tadpole production and the ApaLI transgenic tadpole production.

**Visual Screening.**

DsRed positive tadpoles were determined by observation under a Texas Red filter at 20x magnification with a LEICA DM IRB microscope (Richmond, Illinois). Pictures were taken with an RT slider Spot camera (Figure 1) (Sterling Heights, Michigan).

**Lentiviral Transgenesis**

A second protocol was developed to incubate hatched tadpoles in a solution containing lentivirus overnight to generate transgenic tadpoles. Hormone injections and timing were identical as used to stimulate sperm production and egg laying in the REMI protocol. To induce breeding, a rain chamber was constructed from a 20-gallon aquarium, which was filled halfway with water containing 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts. The water had a hardness (GH or general hardness) of 215 ppm heated to 27°C, and pumped into a container above the aquarium. Water was allowed to drip onto driftwood, a platform, and plastic plants to mimic the wet breeding season. Following
PMSG injection, one to two female *Litoria* were placed into a rain chamber with three to six males. *Litoria* remained in the rain chamber for seven days or until eggs were laid. If eggs were laid, the adult *Litoria* were removed and eggs were allowed to hatch in the rain chamber.

**Lentivirus Production.**

The lentiviral vector chosen for transgenesis was pCDF1 from System Biosciences: a derivative of FIV requiring packaging plasmids for viral production (Mountain View, Ca). The gene isolated for transgenesis was DsRed from the plasmid pCAG, driven by the chicken beta actin promoter. A replication defective lentivirus was generated using system biosciences PEG-it kit (Mountain View, California). The DsRed construct was ligated into pCDF1, a lentiviral vector derived from Feline immunodeficiency virus. Subsequently, the plasmid was transfected into HK 293 cells. Viral pseudoparticles were collected, frozen, and titered using NIH 3T3 cells. Cultures exhibited titers averaging 1.6x10⁶ TU/mL.

**Lentiviral Infection Media.**

Three basal medias were chosen to select for lentiviral media infectivity: Dulbecco’s Modified Eagle Medium (DMEM), NCTC 109, and Leibovitz 15 (L15) medium. NIH 3T3 cells were plated at 1x10⁶ cells/mL in three wells of three 6-well plates in 3 mL of media with 1 µL/mL media of [100 µg/mL] polybrene. A day later cells were infected with replication defective lentiviral particles and observed for fluorescence three days later. Pictures were taken of the wells, and infected versus non-infected cell populations were
recorded. Transformation units were determined between infected and non-infected cell population data (Table 4a). Standard error was calculated and propagated for each well accordingly to give a total standard error for the media. A one-way ANOVA evaluation was performed on the infection populations to determine whether the medias caused the difference in infection rates. Means were separated using least significant difference (LSD) between each media.

*Lentivirus Incubated Tadpole Method.*  
As soon as tadpoles hatched, they were removed from the rain chamber and transported to the laboratory. Procedures were performed under a class II Biosafety Cabinet. Tadpoles were placed into 250 mL beakers with 20 tadpoles per beaker. As a media for the virus and the tadpoles, NCTC 109 media was diluted in half with sterilized water to a volume of 40 mL. To create frog water, 0.1 g/L Seachem Equilibrium and 0.8 g/L Seachem Cichlid Lake Salts was mixed in sterile water. The water had a hardness (GH or general hardness) of 215 ppm. To assist in transduction of viral particles into tadpole cells, 64 µL polybrene [100 µg/mL] was added to the solution. Lastly, 40 mL of virus was added to the solution, making the final ratios half virus, half frog water/NCTC 109 media/polybrene [50 µg/mL]. Tadpoles were incubated in virus for up to 12 hours. Solutions with tadpoles were directly aerated. Tadpoles were observed every 15 minutes.

After incubating in the virus for 12 hours, the tadpoles were moved to containers of the same size with water. When 20 minutes had passed, the tadpoles were moved to their final
destination of 5 large 1.5 liter glass Pyrex bowls. Half of the tadpole water was changed every other weekday with a full change once a week. Tadpoles were cultivated until fully metamorphosed (typically after one to two months), samples of water were incubated with NIH 3T3 cells to ensure tadpoles were not shedding any retrovirus. No DsRed positive cells were observed.

*Litoria Histological Evaluation.*

Histological evaluations were performed on 6 incubated *Litoria caerulea* tadpole tissues. *Litoria* samples were placed in an 80% PBS, 20% sucrose solution. Samples were stored over night at 4°C. A 2:1 solution of 20% sucrose OCT media was prepared as an embedding medium (Tissue-Tek Sakura, AJ Alphen aan den Rijn, The Netherlands). *Litoria* bodies were placed into dry embedding molds, which were filled with embedding medium. The mold was then taken and placed in a small container of methyl butane and floated over liquid nitrogen until the embedding media solidified. Ten micron thick sections were placed on glass slides and mounted in a glycerol based media containing 1 mg/mL p-phenylenediamine (Schwartz et al., 1990). Coverslips were sealed with clear nail polish and samples were observed under a LEICA DM IRB microscope (Buffalo Grove, IL).
RESULTS

REMI De-jelly Results.

Several de-jelly solutions were applied to Litoria eggs to adjust for dehydration (Table 3). A de-jelly solution of 0.5% BSA and 3%-6% Ficoll in 0.4% MMR was found to qualitatively dehydrate Litoria eggs the least. After an adequate de-jelly solution was found, injection solutions were also varied using the optimal de-jelly solutions (Table 2). A solution of 6% Ficoll and 0.5% BSA in 0.4X MMR was found to assist in activating Litoria eggs. As a control injection through the jelly-coat was also attempted on Xenopus and Litoria. Mesh was used to hold the eggs in place and create a single layer of eggs for injection. Injection through the mesh and jelly coat proved difficult; the mesh obscured the location of the poles of the eggs for correct injection site and the eggs would move easily and tear when pierced with the needle. A second problem encountered with this method was the injection medias used typically failed at activating the eggs. As a result few tadpoles were produced and it was determined de-jellying was an easier and more efficient method. The REMI method failed to produce embryo development past neural tube formation in the Litoria species, which may be related to tadpole management.

Lentiviral Infection Media

Cells were plated at 1x10^6 cells/mL per well. Total cell numbers for ten picture sample of the wells were as follows: 31,118 total cells for DMEM, 4,786 total cells for NCTC 109, and 3,937 for L15 media. For three media wells the average transformation units were as
follows; For DMEM media $8.29 \times 10^2$ TU/mL, $1.16 \times 10^5$ TU/mL for NCTC 109 media, and $4.39 \times 10^4$ TU/mL for L15 media were counted (Statistical Methods Table 4b). A one way ANOVA was performed to determine whether there was a significant difference of infection rate among medias: there was significant difference found between DMEM and the other medias, but there is no significant difference between the means of NCTC 109 and L15 (Statistical Methods Table 4b). To confirm the possibility of *Xenopus* cell infection, embryo cells were cultured and transfected with DsRed (Figure 3). Positive cells exhibited fluorescence.

*Xenopus laevis REMI Restriction Enzyme Results.*

The hatchability of REMI transgenic *Xenopus* tadpoles produced using the enzymes SpeI and ApaLI are listed in Table 1. Out of the 825 total tadpoles that hatched, 346 hatched well formed, and 137 expressed the DsRed protein. The total percent of tadpoles that visually expressed the DsRed protein were 22% of the tadpoles produced using SpeI, and 11% using ApaLI. Statistical analysis of an unpaired student t-test was performed on the complete hatchability data but no significant difference was found (Statistical Methods).

Pictures of DsRed-positive developing embryos and hatchlings were taken under incandescent and fluorescent illumination. Under incandescent light developing embryos and hatchlings display pink areas (Figures 1 and 2), under fluorescent illumination.
**Lentiviral Litoria caerulea Microscopy.**

Transgenic *Litoria* tadpoles were successfully generated, cultivated, killed and observed. Samples were collected from transgenic and control froglets one to two months old. Control and Injected tissue sections were examined for DsRed fluorescence under a Texas Red filter (Figures 4 and 5). Tissues observed to be positive for DsRed expression were brain, heart, muscle, and kidney.

**DISCUSSION**

Restriction enzyme mediated integration REMI successfully produced transgenic *Xenopus laevis*. After statistical analysis using an unpaired student t-test, it was found that SpeI and ApaLI are both valid restriction enzymes to use in REMI. Because of the success in the *Xenopus*, the REMI protocol was attempted with *Litoria*. The *Xenopus* hormone injection failed to produce egg laying in the *Litoria*. PMSG was used as hormonal “priming” because it has been shown to increase the total number of early developing oocytes in the ovaries of *Rana cyanophlyctis* without increasing the number of mature oocytes (Pancharatna and Saidapur, 1984). Induction was first attempted in *Litoria caerulea* using a single dose of 1mg/kg LHRH, which previously induced spermatozoa production in the tree frog *Hyla regilla*, with no success (Licht, 1974). It is likely that a single injection of LHRH did not induce egg laying in the *Litoria* because its effects are similar to PMSG; increasing the number of early stage oocytes without increasing maturation or ovulation. A second attempt using PMSG as a hormonal primer and a combination of hCG and Alarelin peptide hormone
(synthetic LHRH) as an inducer, according to a protocol used to induce *Bufo baxteri*, was successful inducing consistent egg laying (Browne et al., 2000). *Litoria* were given hCG as an inducer because it has been shown to shorten oocyte maturation and stimulates ovulation in *Xenopus laevis* (Browne et al., 2000).

REMI was attempted with the jelly coat intact but the injection proved difficult; lower egg activation was observed and proper placement of the injection site was obscured because of the need for mesh to create a single layer of cells. Due to these problems, the de-jelly method was viewed as a more efficient method of production. Several de-jelly and injection medias were attempted to decrease the dehydration of *Litoria* eggs. Solutions containing cysteine free-base appeared to be the most successful in de-jellying the eggs. A higher concentration of cysteine was required to de-jelly *Litoria* eggs than *Xenopus*. These differences contributed to why REMI failed to produce transgenic Litoria. Failure was likely due to a difference between the *Xenopus, and Litoria’s* tolerance to de-jellying the eggs. The jelly coat’s function is to allow attachment to vegetation, provide protection, assist in sperm binding, prevent polyspermy, and act as a barrier to prevent embryo anoxia (Seymour and Bradford, 1995). With removal of the jelly coat, the embryo is more vulnerable to environmental influences.

It has been shown that egg quality and survivability depend on several factors including husbandry conditions of the adult females, water salinity, water contaminants (chlorine, ammonia, nitrate, and nitrite), and general hardness (GH) of the water; with hardness of
water and presence of calcium and magnesium ions being more important than alkalinity or KH (Godfrey and Sanders, 1995). When *Xenopus* eggs were of poor quality, an increase in general hardness increased overall firmness of the vitelline membrane and normal development and survivability of embryos increased (Godfrey and Sanders, 1995). The protocol for maintaining transgenic embryos was modified from the *Xenopus* husbandry protocol to adjust for embryo fragility after de-jellying with an increase in GH. The de-jellying process may affect vitelline membrane elasticity. The vitelline membrane plays a significant role in osmoregulation of the developing embryo (Mild et al., 1973). During the de-jellying process it is possible the vitelline membrane of the *Litoria* eggs was damaged and the general hardness for the de-jellied egg medias needed to be increased further. It is possible that negative results were produced because the husbandry for the *Litoria* tadpoles needed to be adjusted further to account for different salinity, GH, and aeration.

It is also possible during the injection procedure more than one sperm nuclei were injected in some eggs producing abnormal embryo growth. In previous studies, it has been shown that aquatic frogs produce smaller ovum size than the terrestrial species allowing for more oxygenation to the embryo, which assists in faster development (Seymour, 1999). Removal of the jelly coat would influence the embryos ability to respire and could account for slower development/embryo death due to inadequate oxygen/carbon dioxide regulation in the terrestrial species. Although unlikely, polyspermy causes abnormal development in monospermy species.
Transposon transgenesis is a common method used in biotechnology to create transgenic *Drosophila, C. elegans,* and plants (Grabher and Wittbrodt, 2009). The process is similar to REMI involving injection of transposon DNA with synthetic transposase mRNA to cause random insertion into the host genome. Transposon technology does not require a viral backbone like lentivirus transgenesis; the transposon itself is a naturally occurring mobile DNA element (Yergeau et al., 2010). Recently this technology has been applied to vertebrate species, most notably *Xenopus laevis* (Grabher and Wittbrodt, 2009). A major disadvantage to using transposon transgenesis is that its efficiency is inversely related to the inserted transposon’s size, making it an inefficient candidate for large gene insertion (Grabher and Wittbrodt, 2009). Transposon transgenesis also requires species-specific cofactors limiting its application to thoroughly studied species (Grabher and Wittbrodt, 2009). Mosaic expression is common due to the process requiring coinjection of transposase mRNA to catalyze the insertion reaction; the transposase mRNA may not be transcribed before the embryo completes its early stages, meaning the transposase enzyme will not be available to catalyze the reaction in those cells (Yergeau et al., 2010). For these reasons transposon transgenesis was viewed as an inadequate method for this study.

Lentiviral transgenesis produced DsRed positive *Litoria caerulea* tadpoles. The lentivirus vector pCDF1 was driven by the CMV/chicken beta-actin promoter, which drives protein production in the skin, liver, brain, heart, kidney, spleen and lung tissues (Fahim et al., 2009; Lois et al., 2002). Expression was visually confirmed in transgenic tadpole and frog tissues of the brain, heart, kidney, liver, and muscle cells. The distribution of DsRed expression is
similar to studies of GFP lentiviral transgenic mice, where the expression of transgene proteins was found in all tissues and organs analyzed including, skin, bone, muscle, lung, liver, stomach, intestine, kidney, brain, retina and gonads (Lois et al., 2002; Wiznerowicz and Trono, 2005).

Two transgenic species of amphibians were created using the methods of REMI and lentiviral transgenesis. Individuals in the *Xenopus* and *Litoria* species reach maturity at 1-1.5 years of age. Although germline transmission to the F1 generation was beyond the scope of the current study because of the long interval between generations, it is likely that successful germline transmission will occur, as evidenced by the previous success found in chickens, mice, and GFP transgenic *Xenopus laevis* (Lois et al., 2002; Marsh-Armstrong et al., 1999; Mozdziak et al., 2005).
REFERENCES


Table 1. REMI production of transgenic *Xenopus laevis* categorized by restriction enzymes SpeI and ApaLI used on pCAG-DsRed.

<table>
<thead>
<tr>
<th></th>
<th>Restriction Enzyme SpeI</th>
<th>Restriction Enzyme ApaLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent Tadpoles</td>
<td>88</td>
<td>49</td>
</tr>
<tr>
<td>Total Tadpoles</td>
<td>392</td>
<td>433</td>
</tr>
<tr>
<td>Well Formed Tadpoles</td>
<td>129</td>
<td>217</td>
</tr>
<tr>
<td>Percent Fluorescent</td>
<td>22%</td>
<td>11%</td>
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</table>
Table 2. REMI injection solutions for *Litoria caerulea* varied to increase egg activation.

<table>
<thead>
<tr>
<th>Species</th>
<th>Injection Media</th>
<th>Injection Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Litoria caerulea</em></td>
<td>6% Ficoll in 0.1X MMR</td>
<td>Eggs dehydrated, No egg activation</td>
</tr>
<tr>
<td></td>
<td>6% Ficoll in 0.4X MMR</td>
<td>Eggs dehydrated, No egg activation</td>
</tr>
<tr>
<td></td>
<td>0.5% BSA and 6% Ficoll in 0.4X MMR</td>
<td>Eggs dehydrated, Egg activation</td>
</tr>
</tbody>
</table>
Table 3. REMI de-jelly solutions for *Litoria caerulea* and *Xenopus laevis* varied to decrease *Litoria caerulea* egg dehydration.

<table>
<thead>
<tr>
<th>Species</th>
<th>De-jelly Solutions</th>
<th>De-jelly Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Litoria caerulea</em></td>
<td>None- Injection through jelly coat</td>
<td>-Eggs were difficult to inject, none developed</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL Hyaluronidase in 1X MMR</td>
<td>-Eggs dehydrated and not de-jellied</td>
</tr>
<tr>
<td></td>
<td>10% Cysteine free base in sterile water</td>
<td>-Eggs dehydrated and partially de-jellied</td>
</tr>
<tr>
<td></td>
<td>2.5% Cysteine free base in 15% Sb</td>
<td>-Eggs dehydrated and de-jellied</td>
</tr>
<tr>
<td></td>
<td>5% Cysteine free base in 1X MMR</td>
<td>-Eggs dehydrated the least and de-jellied</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>2.5% Cysteine free base in 15% sodium borate (Sb) buffer (10mM NaOH pH to 8.5 with $\text{H}_3\text{BO}_3$)</td>
<td>-Well established method -Eggs dehydrated the least -Majority of transgenic tadpoles produced</td>
</tr>
<tr>
<td></td>
<td>None- Injection through the jelly coat</td>
<td>-Eggs did not activate well/method was difficult -2 tadpoles/100 intact vs. 6 tadpoles /100 de-jellied 100 INJ de-jellied eggs the same day.</td>
</tr>
</tbody>
</table>
Table 4a. Lentiviral titration of 3T3 cells in three medias to determine viral media preference

<table>
<thead>
<tr>
<th>Media</th>
<th>TU/mL</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>8.29x10^2c</td>
<td>4x10^2</td>
</tr>
<tr>
<td>NCTC 109</td>
<td>1.16x10^5a</td>
<td>4x10^4</td>
</tr>
<tr>
<td>L15</td>
<td>4.39x10^4b</td>
<td>1x10^4</td>
</tr>
</tbody>
</table>

ANOVA: Df= 2; P-Value= 0.0069193

There was significant difference found between c and a and c and b, but not between a and b (Table 4b).
**FIG. 1:** Stage 20 DsRed positive *Xenopus* embryo. Positive areas (pink) observed were of the embryos ventral hemisphere (denoted by arrows) (Nieuwkoop and Faber, 1994).
Fig. 2: Stage 20 DsRed positive *Xenopus* embryo. Positive areas (pink) observed were of the embryos ventral hemisphere (denoted by arrows) (Nieuwkoop and Faber, 1994).
**Fig. 3:** Differential interference contrast (A) and fluorescent (B) illumination of cultured DsRed *Xenopus laevis* tadpole tissue. Fluorescence indicates positive results. Scale bar is 30µm.
Fig. 4: CNTL (A, B) and INJ (C, D) tadpole brain tissue viewed under brightfield (A, C) and Texas Red (B, D) filter. Circle indicates positive cerebral tissue.
Fig. 5: CNTL (E, F) and INJ (G, H) tadpole heart tissue viewed under brightfield (E, G) and Texas Red (F, H) filter. Circle indicates positive cardiac tissue.
Fig. 6: CNTL (I, J) and INJ (K, L) tadpole kidney tissue viewed under brightfield (I, K) and Texas Red (J, L) filter. Circle indicates positive renal tissue.
Fig. 7: CNTL (M, N) and INJ (O, P) tadpole muscle tissue viewed under brightfield (M, O) and Texas Red (N, P) filter. Circle indicates positive skeletal muscle tissue.
FIV LENTIVIRAL TRANSGENESIS OF THE LEOPARD GECKO, *EUBLEPHARIS MACULARIUS*

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ABSTRACT: Lentiviral vectors are an effective method of introducing transgenes into the genome of early stage embryos because they transduce both dividing and non-dividing cells. Lentiviral pseudoparticles containing the coding sequence for the fluorescent protein DsRed were injected into freshly laid leopard gecko eggs. Tissue samples were collected from hatchlings, and the samples were tested for presence of the transgene. Of the injected gecko population, 89% of efficiency of transgenesis was confirmed using PCR screening. Histological evaluations revealed the presence of DsRed in injected gecko organs; with protein production concentrated in the umbilical chord, muscle, kidney, heart, and brain. Therefore, lentiviral vectors appear to be viable technology to create transgenic geckos.

Key words: DsRed; *Eublepharis macularius*; FIV; Lentiviral transgenesis; Leopard gecko

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LENTIVIRUSES are used in biotechnology to integrate foreign DNA into a host genome, facilitating foreign gene expression (Pfeifer and Hofmann, 2009). Lentiviruses belong to the family of retroviruses, but unlike most retroviruses; they transduce both dividing and non-dividing cells in vivo (Cockrell and Kafri, 2007). The first lentiviral vectors used for transgenesis were products of the HIV family, consisting of three vectors coding for viral particle generation (Nakagawa and Hoogenraad, 2011; Pfeifer and Hofmann, 2009).

Development of FIV based lentiviral vectors began partly because FIV does not cause human infection. The FIV genome is simpler than the HIV genome containing only 3 non-structural genes, but is similar to other lentiviruses in genome structure and morphology (Saenz and Poeschla, 2004). FIV based vectors have been used to transduce non-dividing and dividing cells of the brain, eye, airway, hematopoietic system, liver, muscle, and pancreas (Curran et al., 2002; Derksen et al., 2002; Loewen et al., 2001; Price et al., 2002; Stein and Davidson, 2002; Wang et al., 1999). Lentivirus transgenesis has been used to produce transgenic species including mammals (mouse, rat, pig, bovine), avian, fish and various amphibians (Nakagawa and Hoogenraad, 2011; Pfeifer and Hofmann, 2009). PCDF1 based vectors have produced positive results in mammalian cells (SBI, Mountain View, Ca).

Fluorescent proteins (FPs) occur naturally in organisms of four phyla (Cnidaria, Ctenophora, Arthropoda, and Chordata) including jellyfish, crustaceans, comb jellies, and chordates (Chudakov et al., 2009; Deheyn et al., 2007; Haddock and Case, 1999; Shagin et al., 2004). DsRed, a 28-kDa red homologue of the fluorescent protein GFP, is isolated from the Discosoma species of coral (Matz et al., 1999). The DsRed fluorescent protein has a 583nm
emission wavelength within the visual spectrum (Sakaue-Sawano et al., 2008; Strack et al., 2010). DsRed is an oligomeric fluorescent label that has been used extensively in research involving microscopy and flow cytometry (Strack et al., 2010).

The objective of this study was to achieve lentiviral mediated *Eublepharis macularius* transgenesis. Leopard geckos are produced in large numbers because they are easy to manage in captivity (De Vosjoli et al., 2005; Thorogood and Whimster, 1979; Wise, 1997; Wise et al., 2009). Because of the leopard gecko’s popularity, captive bred lines are available negating the need to obtain wild caught individuals. Captive bred lines of leopard geckos also demonstrate a range of morphological differences including pattern variations such as jungle, striped, patternless, and color variations such as tangerine, white, lavender, melanistic, amelanistic, and leucistic (De Vosjoli et al., 2005). In previous studies, leopard geckos have been used as models for reproductive physiology, central nervous system development, tissue regeneration, and studies of gene expression (Bull, 1987; Valleley et al., 2001; Whimster, 1978). Leopard geckos were chosen as the species for transgenesis because of their general hardiness, captive bred genetic lines, and lastly because their eggs are soft shelled and measure 25 x 12 mm on average; making them attractive for injection of lentiviral particles into the embryos (Minton, 1966).
MATERIALS AND METHODS

Lentivirus Production

The lentiviral vector chosen for transgenesis was pCDF1 from System Biosciences: a derivative of FIV requiring packaging plasmids for viral production (Mountain View, Ca). The gene isolated for transgenesis was DsRed from the plasmid pCAG, driven by the chicken beta actin promoter. A replication defective lentivirus was generated using system biosciences PEG-it kit (Mountain View, California). The DsRed construct was ligated into pCDF1, a lentiviral vector derived from Feline immunodeficiency virus (Fig. 1). Subsequently, the plasmid was transfected into HK 293 cells. Viral pseudoparticles were collected, frozen, and titered using NIH 3T3 cells. Cultures exhibited titers averaging 1.6x10^6 TU/mL.

Gecko Egg Injection:

The Gourmet Rodent INC supplied the gecko eggs (Jonesville, FL). All procedures involving animals were approved by the NC state Institutional Animal Care and Use Committee. Geckos were killed for sampling by an overdose in sodium pentobarbital. Eggs were illuminated under brightfield to ensure fertility and correct oviposition. Lentivirus particles were applied to gecko embryos at stage 29 of development by inserting a 27-gauge needle below the blood ring of the developing embryo and injecting ~200 µL of lentiviral supernatant with 100 µg/mL polybrene (Wise et al., 2009). Liquid cement was used to seal the hole. Eggs were incubated in soaked Perlite (The Scotts Company, Marysville, OH) at
temperatures between 27-31°C. Control (CNTL) and injected (INJ) eggs hatched after 30 days of incubation. To ensure that helper retrovirus was not present in the INJ embryos, INJ geckos were macerated and applied to NIH 3T3 cultures, which were observed 2 days later. No positive cells were observed.

Gecko Observation:
Immediately after hatching geckos were observed using a Night Sea DFP-1 Dual Fluorescent Protein flashlight (Bedford, Ma). Pictures of the INJ geckos were taken using a Canon Rebel digital camera (Pleasant Prairie, WI).

Polymerase Chain Reaction:
DNA Extraction Materials. -DNA was isolated from 9 INJ geckos and 5 CNTL geckos. Bodies were divided in half; the caudal half, from below the liver to the tail, was digested using the QIAGEN DNA easy kit and analyzed using PCR, the dorsal half was preserved for histological evaluations (Valencia, California). Samples were macerated, cells were lysed, and DNA was extracted. DNA concentrations were evaluated by measuring the 260/280 ratio using a Thermo Scientific nanodrop (Wilmington, DE). PCR was performed on the 9 gecko DNA samples to confirm presence of DsRed DNA in the samples. Primer sequences are listed below.
PCR reactions were executed using PROMEGA GoTaq Green Master Mix, 10μM forward and reverse primers, and 1μg sample DNA (Madison, Wisconsin). An annealing temperature of 62-65°C was used for all primers. Appropriate temperatures were achieved using a Bio-Rad MJ research Peltier Thermal Cycler 200 (Ramsey, Minnesota). DNA samples were amplified using primers that were expected to generate a 711 bp fragment of the DsRed gene. PCR reactions were then fractionated to 1% agarose gels for examination. Bands indicative of positive results were excised from the gels and purified using a Qiagen Gel Purification kit.

Subsequently, the DNA was reamplified using DsRed primers that amplify a nested 169 bp segment internal to the 711 bp fragment. Amplification products were verified as DsRed by sequencing (Eton BioScience Durham, NC).

**Histological Evaluation:**

Dorsal half of gecko samples were placed in an 80% PBS, 20% sucrose solution. Samples were stored over night at 4°C. A 2:1 solution of 20%sucrose OCT media was prepared as an embedding medium (Tissue-Tek Sakura, AJ Alphen aan den Rijn, The Netherlands). Gecko
bodies were placed into dry embedding molds, which were filled with embedding medium. The mold was then taken and placed in a small container of methyl butane and floated over liquid nitrogen until the embedding media solidified.

Ten micron thick sections were placed on glass slides and mounted in a glycerol based media containing 1 mg/mL p-phenylenediamine (Schwartz et al., 1990). Coverslips were sealed with clear nail polish.

RESULTS

Hatchability and Incubation:
Hatchability of the injected geckos was 36.6% versus 70% in the CNTL group (Table 1). Statistical analysis of an unpaired student t-test was performed on the complete hatchability data (Statistical Methods).

Visual Screening:
Hatched INJ geckos were observed under fluorescent illumination. Geckos exhibited a mosaic of visual fluorescence. All observed exhibited fluorescence (Figures 2 and 3).

PCR Screening:
DNA samples were amplified using primers that would be expected to generate a 711 bp fragment (Figure 4). Subsequently the 711 bp band was amplified using PCR primers internal
to the 711 band (Figure 5). Out of the INJ population, 9 samples of DNA were randomly selected for PCR testing. Out of the 9 tested, 8 were found to be positive using nested PCR. An 89% overall success rate for success of the presence of the gene was achieved.

**Microscopy:**

Tissue sections were examined for DsRed fluorescence under a Texas Red filter at 40x magnification (Figure 6) and 20x magnification (Figure 7). Tissues observed to be positive for DsRed expression were muscle, kidney, heart, and brain.

**DISCUSSION**

A single injection of lacZ gene retroviral particles to chicken eggs was an efficient method of transgenesis; producing more transgenic chickens than a method where more than one injection was used (Mozdziak et al., 2003). Hatchability between INJ and CNTL geckos was compared using a student's t-test; the calculated p-value=0.06 was greater than alpha= 0.05. This lead to the conclusion that it is probable the variability in hatchability was not significant and the difference in hatchability between groups was caused by randomness. However, the total number of injected eggs was greater than the total number of control eggs (112 INJ eggs total vs 20 CNTL eggs total). It is possible the control population was not large enough to be a successful control comparison. Similar studies in chickens showed the act of egg injection and insertion of viral particles into an egg lowers hatchability: fully intact eggs reached a 67.9% hatchability, injected eggs (no virus) hatchability was 56.8%, and
when injecting concentrated retroviral particles the hatchability dropped to below 30% (Bednarczyk et al., 2000; Harvey et al., 2002). Chi squared tests on this data revealed a significant difference among groups (Bednarczyk et al., 2000). The difference in chicken egg hatchability is consistent with the gecko egg hatchability data of 70% of CNTL and 36.6% of INJ geckos hatched (Table 1). It is likely hatchability decreases because the hole in the shell also opens the embryo to a greater possibility of infection from mold and bacteria, and makes the embryo more subject to temperature and humidity fluctuates. Embryos can also be damaged physically from a piercing from the needle causing malformations. The success of lentiviral transgenesis is reliant on the high titer of virus, and availability of the virus (Gama Sosa et al., 2009).

Vector pCDF1 is driven by the CMV/chicken beta-actin promoter, which drives protein production in the skin, liver, brain, heart, kidney, spleen and lung tissues (Fahim et al., 2009; Lois et al., 2002). Leopard gecko production of DsRed transgenic proteins were found in muscle, heart, kidney, liver, and brain. The distribution of DsRed expression is similar to studies of GFP lentiviral transgenic mice, where the expression of transgene proteins was found in all tissues and organs analyzed including, skin, bone, muscle, lung, liver, stomach, intestine, kidney, brain, retina and gonads (Lois et al., 2002; Wiznerowicz and Trono, 2005.). Lentiviral transgenesis is a forthcoming method of creating transgenic lines. Expressing transgenic F1 progeny have been produced in mice using a similar method of lentiviral transduction (Lois et al., 2002; Nakanishi et al., 2002). Leopard geckos reach breeding age after 1-2 years, or when they reach 40 grams (De Vosjoli et al., 2005). Although germline
transmission to the F1 generation was beyond the scope of the current study because of the long interval between generations, it is likely that successful germline transmission will occur, as evidenced by the previous success found in chickens, mice, and *Xenopus laevis* (Lois et al., 2002; Marsh-Armstrong et al., 1999; Mozdziak et al., 2005).
FIV LENTIVIRAL TRANSGENESIS OF THE LEOPARD GECKO

LITERATURE CITED


LENTIVIRAL TRANSGENESIS TABLES

Table 1.- Hatchability between injected (INJ) and control (CNTL) gecko eggs.

<table>
<thead>
<tr>
<th></th>
<th>Eggs Hatched</th>
<th>Total Eggs</th>
<th>% Hatchability</th>
</tr>
</thead>
<tbody>
<tr>
<td>INJ Gecko Eggs</td>
<td>41</td>
<td>112</td>
<td>36.6%</td>
</tr>
<tr>
<td>CNTL Gecko Eggs</td>
<td>14</td>
<td>20</td>
<td>70%</td>
</tr>
</tbody>
</table>

Table 2.- PCR detection of DsRed in leopard gecko hatchlings. Of the 41 hatched injected geckos, 9 total geckos were tested.

<table>
<thead>
<tr>
<th>Total Geckos</th>
<th>% Positive Geckos</th>
<th>% Negative Geckos</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>89%</td>
<td>11%</td>
</tr>
</tbody>
</table>
FIGURES AND FIGURE LEGENDS

pCDF1-MCS2-EF1-Puro Vector

CMV/5’LTR: Hybrid CMV promoter-R/U5 long terminal repeat
Gag: Packaging signal
RRE: Rev response element
cPPT: Central polypurine tract
CMV Promoter: Human cytomegalovirus promoter
Multiple Cloning Site 1 (MCS1)
WPRE: Woodchuck hepatitis virus posttranscriptional regulatory element
3’LTR: Self-inactivating 3’LTR
SV40 Poly-A: Transcription termination and polyadenylation
SV40 Ori: Episomal replication of plasmid
pUC Ori: High-copy replication
AmpR: Ampicillin Resistance gene

FIG. 1: pCDF1-MCS2 lentiviral backbone vector construct. Construct includes CMV promoter, sequences for the proteins gag, RRE, cPPT, multiple cloning site (MCS1), woodchuck hepatitis virus posttranscriptional regulatory element, 3’long terminal repeat promoter (LTR), SV40 Poly-A termination signal, SV40 origin, pUC origin, and ampicillin resistance gene.
pCDF1-DsRED Vector: 1-6128bp
pCDF1 CAG-CMV Promoter: 18-1718bp
pCDF1 Chicken β-Actin Promoter: 382-661bp
pCAG DsRed Insert: 1720-2448bp
pCDF1 Multiple Cloning Site 1: 2449-2559bp
pCDF1 Vector Continues: 2449-6128bp

**FIG. 2:** Location of cloned DsRed insert, CAG- Chicken beta-actin promoter, and CAG-Cytomegalovirus promoter, in the vector pCDF1-DsRed vector.
Fig. 3: CNTL (A, B) and INJ (C, D) gecko hatchlings viewed under fluorescent illumination. Arrow heads indicate organ fluorescence, arrows indicate punctate fluorescence spots in skin. Scale bar is 1cm.
Fig. 4: 711 Amplification of intact (CNTL) and injected gecko (INJ) DNA. Lane 1: 1kb ladder, Lane 2: H₂O control, Lane 3: CNTL gecko DNA, Lane 4: INJ 1, Lane 5: INJ 2, Lane 6: Positive control DNA from DsRed infected 3T3 cells.

Fig. 5: 169 Amplification of CNTL and INJ gecko DNA from excised 711 gel bands. Lane 1: 1kb ladder, Lane 2: H₂O control, Lane 3: Intact gecko DNA, Lane 4: Injected gecko 1, Lane 5: Injected gecko 2, Lane 6: Positive control DNA from DsRed infected 3T3 cells.
Fig. 6: 40x magnification CNTL (A, B) and INJ (B, D) gecko muscle viewed under brightfield (A, C) and Texas Red filter (B, D). Scale bar is 30µm.
FIG. 7: CNTL (A, B) and INJ (C, D) gecko brain tissue viewed under brightfield (A, C) and Texas Red (B, D) filter.
Fig. 8: CNTL (E, F) and INJ (G, H) gecko heart tissue viewed under brightfield (E, G) and Texas Red (F, H) filter.
Fig. 9: CNTL (I, J) and INJ (K, L) tadpole kidney tissue viewed under brightfield (I, K) and Texas Red (J, L) filter.
Litoria caerulea, and Xenopus laevis REMI

The purpose of these studies was to produce transgenic *Litoria caerulea, Xenopus laevis*, and *Eublepharis macularius* to investigate laboratory use of the species and to gain insight in the use of REMI and lentiviruses as valid methods of transgenesis. Using restriction enzyme mediated integration (REMI) transgenic *Xenopus laevis* were successfully produced. Because of the success in *Xenopus*, the REMI protocol was applied to *Litoria*. Problems encountered during the procedure included an inability to induce egg laying in the land-dwelling frog species *Litoria caerulea* with hormones used in *Xenopus laevis*, and dehydration of the *Litoria* eggs after a de-jellying solution was applied. Several hormonal solutions were implemented and egg laying was eventually accomplished with species-specific solutions. REMI was unsuccessful in producing fully developed *Litoria* embryos; development typically halted at or before neurulation. The cause of early embryo death is most likely damage the eggs received from the procedure. Adjustments may need to be made to the husbandry of the injected eggs to further protect them from environmental damage after de-jellying. Further studies of the successful *Xenopus laevis* include breeding the transgenic F0 generation to each other to confirm germline transmission of the DsRed gene. Although germline transmission to the F1 generation was beyond the scope of the current study because of the long interval between generations, it is likely that successful germline transmission will occur, as evidenced by previous REMI success found in GFP transgenic *Xenopus laevis* (Marsh-Armstrong et al., 1999).
Eublepharis macularius and Litoria caerulea Lentiviral Transgenesis

Two novel methods of lentiviral transgenesis were performed on Litoria caerulea and Eublepharis macularius. Lentiviral transgenesis was successful in producing DsRed transgenic Litoria and Eublepharis. Expression was visually confirmed in transgenic frog and gecko tissues of the brain, heart, kidney, and muscle cells. Hatchability between control and injected groups of geckos varied. Insertion of viral particles into an egg has been shown to lower hatchability. When similar procedures were performed on chicken eggs those injected with concentrated retroviral particles the hatchability dropped to below 30% versus 56.8% in eggs where a small injection hole was made (Harvey et al., 2002). Leopard geckos reach breeding age after 1-2 years, or when they reach 40 grams so germline transmission was beyond the scope of this study (De Vosjoli et al., 2005). Germline transmission by lentiviral transgenesis has been shown in mice, chickens, and non-human primates so it is likely with further experimentation germline transmission will be proven in the leopard gecko and White’s tree frog (Lois et al., 2002; McGrew et al., 2004; Sasaki et al., 2008).


STATISTICAL METHODS SECTION

REMI Transgenesis Xenopus Hatchability T-Test Evaluation

Hatchability of Xenopus tadpoles using restriction enzymes Spe I and Apa LI was examined using an unpaired student t-test. A 95% confidence interval for means was applied to the data; p-value=0.3714 >0.05 fail to reject null hypothesis. On average, the reported relationship between Spe I (M=24.50, SD=17.87) were not significantly different than Apa LI (M=33.31, SD=33.41), df=27, t=0.9091, p=0.3714. Restriction enzymes Spe I and Apa LI are both valid enzymes to use on the pCAG DsRed plasmid for REMI transgenesis.

Table 4b. ANOVA table of lentiviral titration of 3T3 cells in three medias to determine viral media preference

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>1.15e14</td>
<td>2</td>
<td>5.74e13</td>
<td>12.7</td>
<td>0.00692</td>
<td>5.14</td>
</tr>
<tr>
<td>Within Groups</td>
<td>2.70e13</td>
<td>6</td>
<td>4.50e12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.42e14</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Statistical analysis of ANOVA was applied to the lentiviral titration data for three medias. Significant difference was found between DMEM and NCTC 109 and DMEM and L15, but there is no significant difference between the means of NCTC 109 and L15. NCTC 109 media was chosen for transgenesis.
Lentiviral Transgenesis Gecko Hatchability T-Test Evaluation

Gecko hatchability between INJ and CNTL gecko populations was examined using an unpaired student t-test. A 95% confidence interval for means was applied to the data; p-value =0.06>0.05 fail to reject null hypothesis. On average, the reported relationship between hatched INJ gecko eggs (M=5.12, SD=4.45) were not significantly different than hatched CNTL gecko eggs (M=1.75, SD=1.67), df= 14, t= -2.01, p=0.064. It is probable the variability in hatchability was not significant and the difference in hatchability between groups was caused by randomness.