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ASSISTED BREEDING OF SKINKS OR HOW TO TEACH A LIZARD OLD TRICKS!

FRANK C. MOLINIA[1], TRENT BELL[2,3], GRANT NORBURY[4], ALISON CREE[5] AND DIANNE M. GLEESON[1]

[1]Landcare Research, Private Bag 92170, Auckland 1142, New Zealand,e-mail: molinia f@landcareresearch.co.nz
[2]Landcare Research, Private Bag 1930, Dunedin 9054, New Zealand
[3]Current Contact Details: EcoGecko Consultants, e-mail: trent@ecogecko.co.nz
[4]Landcare Research, PO Box 282, Alexandra 9340, New Zealand
[5]Department of Zoology, University of Otago, PO Box 56, Dunedin 9054, New Zealand

***Abstract.*—Reproductive technologies are invaluable tools for understanding how different species reproduce. Contemporary techniques like artificial insemination established long ago in livestock have been used to assist the breeding of threatened species *ex situ*, even restoring them to nature. Key to successfully adapting these technologies, often to few numbers of endangered animals, is initial testing and development of procedures in a taxonomically related model species. McCann's Skink (*Oligosoma maccanni)* is a viviparous lizard that is still relatively abundant and its reproductive cycle in the subalpine area of Macraes Flat in southern New Zealand has recently been described. Assisted breeding techniques are being developed in this skink as a model for threatened lizard species, such as the Grand Skink (*Oligosoma grande)* and Otago Skink (*Oligosoma otagense*). Progress on methods to collect, assess and store sperm, and artificial insemination are reported here. These techniques will need refinement to be effectively adapted to threatened lizards but will significantly increase our knowledge of their unique reproductive mechanisms. In the longer term they are expected to improve substantially captive breeding success and will be vital tools to aid genetic management of animals bred for release to restored ecosystems and secure genetic repositories for future restoration needs.**

Key Words.—artificial insemination; assisted breeding; conservation; genetic management; *Oligosoma* species; reproductive technologies; skink sperm; threatened lizards

OVERVIEW

In recent years there have been reports in the media and scientific press that human beings are currently causing the greatest mass extinction of species since the extinction of dinosaurs 65 million years ago, and if present trends continue, one half of all species on earth will be extinct in less than 100 years as a result of habitat destruction, pollution, invasive species, and climate change (Novacek and Cleland 2001; Thomas et al. 2004; Lewis 2006; Brook et al. 2008; Vie et al. 2009). It is difficult to ascertain how precarious the situation may be globally for reptiles. The latest figures indicate that approximately 28% of all reptile species evaluated are threatened, although fewer than 19% of all reptile species described have been comprehensively evaluated to date (IUCN, Conservation International, and NatureServe. 2009. IUCN Red List of Threatened Species. Version 2009.2. Available from http://www.iucnredlist.org [Accessed 9 November 2009]). Reptiles exhibit a bewildering array of reproductive modes (including temperature sex-determination, parthenogenesis, and viable sperm retention in the female tract for up to five years), yet so little information on the physiology and hormonal control of reproduction exists (Lance 2003). Reproduction is core to species survival, so understanding how an animal breeds is fundamental to conserving species, populations, and, indirectly, the vitality of entire ecosystems (Wildt et al. 2003). In the face of growing species extinctions the need for research that addresses these knowledge gaps is urgent. With that objective in mind, this paper will articulate how emerging reproductive technologies are being developed to assist the conservation effort of threatened New Zealand lizards. A brief description of what reproductive technologies are, which ones are appropriate, why they are valuable, and the importance of 'model' species for technology development is presented including progress to date in reptiles. This will preface discussion of the suitability of using McCann's Skink *(Oligosoma maccanni*; Fig. 1) as a model to develop assisted breeding techniques for target threatened species like the Grand Skink (*Oligosoma grande)* and Otago Skink (*Oligosoma otagense*). All three species are endemic to New Zealand, where protection of lizards is given a high priority for government and community conservation programs. We will publish elsewhere a comprehensive description of the experimental design, protocols examined to collect, assess, and store sperm, and artificial insemination methodology at the conclusion of the study. An update

FIGURE 1. McCann's Skink *(Oligosoma maccanni).* (Photograph courtesy of Trent Bell)

on technology development in the model species will be reported here. An assessment will follow on the expected outputs, implications, and likely outcomes that this research could have for conservation of target threatened species should the techniques be successfully adapted.

REPRODUCTIVE TECHNOLOGIES AND WILDLIFE 'MODEL' SPECIES

The study of reproductive science includes all skills required to address priorities for understanding, monitoring, enhancing, and controlling reproduction (Wildt et al. 2003). Reproductive technologies are a subset discipline under the reproductive sciences umbrella and collectively refer to techniques that contribute to an understanding of what regulates reproductive success (Pukazhenthi and Wildt 2004). However, from the outset it is important to realize that reproductive technologies are just one piece of a complex puzzle to the achievement of conservation outcomes (Wildt et al. 2001, 2003). For example while reproductive technologies were valuable for propagating the endangered Black-Footed Ferret (*Mustela nigripes*) in captivity, the successful re-introduction of offspring of known provenance and appropriate genotype to preserve integrity and ensure persistence *in situ* required a conservation-effective approach. This drew on the integration of many scientific disciplines with specific tools and skills in partnership with those of stakeholders, sociologists, economists, demographers, and wildlife/habitat managers themselves (Howard et al. 2003).

Reproductive technologies range from low-technology methods like behavioural observations of reproductive activity and non-invasive endocrine monitoring to high-technology approaches like intracytoplasmic sperm injection, cloning, and stem cell-based technologies. There is much debate over which of the latter emerging technologies will be most useful for the conservation of threatened species in the future (Pukazhenthi et al. 2006), but there is little argument that the foremost value of reproductive technologies are as tools for studying how different species reproduce, especially defining novel and unique mechanisms (Pukazhenthi and Wildt 2004). Impressive progress has been made over the last 60 years to assist reproduction of livestock, domestic and laboratory animals, and humans through the use of artificial insemination, *in vitro* oocyte maturation and culture, *in vitro* fertilization, embryo transfer, and germplasm banking. Currently, only non-invasive hormone monitoring, artificial insemination, and sperm banking have been used routinely for genetically managing wildlife species *ex-situ* and even restoring species to nature, which is precisely why these 'old tricks' are being developed for New Zealand lizards.

Key to the success of adapting reproductive technologies is the use of 'model' wildlife species (Wildt et al. 1986, 2001; Pukazhenthi et al. 2006). Often the target species is already threatened so there are simply too few animals to develop the technology directly. Given overwhelming failure of the 'quick-fix' approach, where it was naively assumed that technology developed in livestock or humans could readily propagate rare wildlife, it is now clear that reproductive mechanisms are species-specific, and that initial testing and application is appropriate in a taxonomically related model species (Wildt et al. 2001). For example, reproductive technologies like non-invasive hormone monitoring, sperm collection, processing, and storage, and artificial insemination have been developed in the Domestic Ferret *(Mustela putorius furo)* for the Black-Footed Ferret (reviewed by Howard et al. 2003), the Domestic Cat *(Felis catus)* for wild felids (e.g., Brown 2006;; Pelican et al. 2006), and the Brushtail Possum *(Trichosurus vulpecula)* and Tammar Wallaby *(Macropus eugenii)* for endangered marsupials (eg., Molinia et al. 2007; Rodger et al. 2009). Even then, a technique that works efficiently in the model will invariably require further modification to be successfully adapted in the target species with its own unique reproductive vagaries. The basal information obtained from models though will mean that adapting a technique for target species will be safer and success more likely.

There has been relatively modest development of reproductive technologies in herpetofauna species compared with the vast literature in other vertebrates. However, over the last decade there has been significant advances in frog assisted breeding techniques, including urinary hormone analysis to non-invasively monitor reproduction and assign sex of individuals (Germano et al. 2009; Narayan et al., 2010), the use of exogenous hormones for induction of spermiation and ovulation, *in* *vitro* or artificial fertilization, short-term cold storage of gametes and long-term cryopreservation of spermatozoa for gene banking (reviewed by Kouba et al. 2009; Kouba and Vance 2009) that are now being used for the conservation of threatened or endangered amphibian species. In reptiles though, much less progress has been made. Detailed endocrine and reproductive physiological information has been collected from a few reptile species predominately those in temperate zones (reviewed by Lance 2003), but most studies have involved invasive procedures like repeated blood sampling or indeed harvest of animals. Female reproductive condition has been evaluated using a range of techniques from non-destructive methods like laparoscopy (e.g in tuatara, Cree et al. 1991) to non-invasive methods like radiography, ultrasound, and palpation (e.g. reviewed by Lance 2003; Holmes and Cree 2006). A combination of procedures has been suggested to gain most information throughout the reproductive cycle (e.g. Gartrell et al. 2002), although ultrasound alone could determine seasonal reproductive changes in the Mexican viviparous lizard *Barisia imbricata* including follicular development and ovulation (Martinez-Torres et al. 2006). Alternatives to invasive blood sampling, which could be prohibited on practical and welfare grounds in endangered species (Pickard 2003), have also been tested in reptiles. The sex of hatchling Loggerhead Turtles *(Caretta caretta)* could be identified using measures of testosterone and oestradiol metabolites in egg chorioallantoic/amniotic fluid (Gross et al. 1995). Fecal glucocorticoids as an indicator of stress have been developed for turtles to evaluate some common management procedures like fitting radio transmitters (Rittenhouse et al. 2005) and enrichment of a captive environment (Case et al. 2005). A fecal testosterone assay has also been developed for Blue-Tongued Lizards *(Tiliqua nigrolutea)*, but due to the lack of correlation with plasma samples, was not useful for assessing reproductive status of males (Atkins et al. 2002).

As reviewed by Millar and Watson (2001), semen collection in live animals has been achieved using a massage technique in snakes (and recently adapted in geckos, Todd 2003), and was more reliable than electroejaculation. In alligators electroejaculation or chemical stimulation of ejaculation has been used to collect semen, but similar numbers of sperm could be retrieved relatively non-invasively following aspiration of sperm from the penile groove, while electroejaculation remains the best method for obtaining sperm from chelonians (Millar and Watson 2001). Epididymal sperm collected post mortem has been used to develop diluents that best support sperm motility in lizards (reviewed by Millar and Watson 2001) and turtles, where sperm incubated at 2° C could survive in excess of 40 days *in vitro* (Gist et al. 2000, 2001). Sperm preservation and artificial insemination studies have been conducted in the American Alligator *(Alligator mississippiensis)*, and while artificial insemination has been successful it required the use of fresh spermatozoa recovered post-mortem (Larsen et al. 1984, 1988). Semen extenders have been developed for the Broad-nosed Caiman *(Caiman latirostris)* using sperm aspirated from the penile groove of live males (Larsen et al. 1992), but no successful artificial insemination has been reported. In snakes basic seminal quality and sperm motility parameters have been established in model species like the free-ranging Brazilian Rattlesnake (*Crotalus durissus terrificus*; Zacariotti et al. 2007), and selected threatened species such as the Argentine Boa Constrictor *(Boa constrictor occidentalis*; Tourmente et al. 2007) ahead of developing semen cryopreservation and artificial insemination technology. Evaluation of ejaculate traits has even been used as a tool for better understanding the mechanisms of reptile fertilization such as sperm competition in the Northern Watersnake (*Nerodia sipedon*; Schulte-Hostedde and Montgomerie 2006). Specialist media have been developed for both cooled and frozen storage of snake sperm (reviewed by Millar and Watson 2001; Fahrig et al. 2007), and artificial insemination has been successful using fresh electroejaculated semen in the Checkered Garter Snake (*Thamnophis marcianus*; Quinn et al. 1989) and in the Corn Snake (*Elaphe gutatta*) with fresh and cooled semen collected non-invasively using the massage technique (Mattson et al. 2007).

While some progress has been made it is clear there is still much scope for development of reproductive technologies in reptiles. Apart from New Zealand lizards detailed in the present study, another emerging candidate species for such technology development is the tuatara (*Sphenodon* sp.). Recently the mating system of a captive-bred (Moore et al. 2008) and wild population on Stephens Island (Moore et al. 2009) was explored revealing a low incidence of multiple paternity and male reproduction was highly skewed with few males dominating mating. This raises interesting questions about fertilization mechanisms and sperm competition in this species, let alone the need for methods to offset the risk of future inbreeding and genetic bottleneck. This could be addressed through development of new technologies like a reliable non-invasive method to collect and analyse sperm along with artificial insemination (Moore 2008). Clearly reproductive technology development in reptiles in the near future will be driven by the need for this type of basic knowledge about unique reproductive strategies, let alone techniques to help manage genetic diversity of dwindling populations as more species become threatened with extinction.

FIGURE 2. Sperm collection from McCann's Skink (*Oligosoma maccanni*). Semen that accumulates between the hemipenes after lower abdominal massage is collected by capillary action into a small glass microtube. (Photographed by Trent Bell)

FIGURE 3. Artificial insemination of McCann's Skink (*Oligosoma maccanni*). Diluted sperm is deposited into each lateral side of the cloaca using a pipette. (Photographed by Trent Bell)

NEW ZEALAND LIZARDS AND REPRODUCTIVE TECHNOLOGY DEVELOPMENT

The majority of New Zealand's herpetofauna is endemic and includes four extant species of native frog, two species of tuatara, and over 80 species of lizards comprised of geckos and skinks. The threatened status of New Zealand reptiles and amphibians is currently being reviewed, but at the last assessment more than 50% of lizards were classified as rare, threatened, or endangered (Hitchmough et al. 2007). Two of New Zealand's largest and rarest lizards are the Grand Skink and the Otago Skink, which inhabit schist rock outcrops in montane tussock grassland in Otago, southern New Zealand. They currently occupy only 8% of their former range as two separate eastern and western populations, and mammalian predation and habitat loss are thought to be the main causes of their decline (Norbury et al. 2006). Classified as 'Nationally Critical' since 2003, recent population monitoring and modelling suggests a high probability of functional extinction for both species by 2010 (Tocher and Norbury 2005). Both skinks are diurnal, long lived (> 16 years in the wild), viviparous, and slow to reach sexual maturity (offspring produced after 4 years). In the subalpine area of Macraes Flat, both species appear to breed annually with vitellogenesis during autumn-spring, a gestation of 4–5 months, and parturition in summer resulting in an average of around two offspring produced per female (Cree 1994; Tocher 2009). However, definitive studies are not possible due to the now endangered status of these species.

Reproductive technologies and specifically assisted breeding techniques are being developed to improve knowledge of the reproductive biology of the Grand Skink and the Otago Skink. There also is the hope to achieve the long-term goal of the recovery program to maintain and restore viable populations of both skink species across their natural range, and to maintain their genetic diversity (Norbury et al. 2006). McCann's Skink occurs sympatrically with these target species, is taxonomically related, and is still relatively abundant. They are viviparous lizards that also breed annually and the length of vitellogenesis and pregnancy is similar while ovulation and parturition occur earlier than the Grand Skink and Otago Skink (Holmes and Cree 2006). It is for these reasons that they were chosen as a suitable model species to develop assisted-breeding techniques for the target threatened lizards.

To date, significant progress has been made on collection, assessment, and short-term (liquid) storage of sperm, and development of an artificial insemination technique in McCann's Skink. Adapting a lower abdominal massage technique developed in the Common Gecko (*Hoplodactylus maculatus*; Todd 2003), microlitre volumes of semen could be reliably collected from male McCann's Skink during the peak of their mating period (March; Fig. 2). Methods have been standardized for evaluating sperm quality, including assessments of semen volume, sperm concentration, and motility. Several buffers were tested for their suitability to support sperm motility after short-term (liquid) storage. The highest motility was obtained following dilution in a medium used for turtle sperm (Gist et al. 2000) where more than 70% of McCann's Skink sperm were still motile after five days incubation at 4° C. Artificial insemination of females was developed based on a cloacal sperm deposition technique established for birds (reviewed by Donoghue et al. 2003; Fig. 3). Given that McCann's Skink mates in autumn and ovulate in spring (Holmes and Cree 2006), their sperm must be stored in the female reproductive tract for several months during vitellogenesis. This means that there is likely a large window of opportunity to inseminate females using collected sperm.

To mimic natural mating, 10 females were inseminated in March 2008. Pooled sperm from six males collected over two consecutive days was used to introduce two 5 uL doses of diluted sperm deep into each lateral side of the cloaca, such that each female received at least 1 x 10^6 motile sperm. During May 2008, eight females were confirmed to be cycling due to the detection of at least one vitellogenic follicle per female after gently palpating the abdominal cavity between finger and thumb (Holmes and Cree 2006). In December 2008, about half of the females appeared gravid, but unfortunately a parasitic disease outbreak occurred over the early New Year period that resulted in loss of half the colony. Those lizards remaining were maintained in their outdoor cages and thus exposed to normal photoperiod in Dunedin, but no births were recorded by March 2009. In McCann's Skink it is now known that the correct thermal environment is vital for pregnancy success (Cree and Hare 2010) and parasites have also been implicated in pregnancy failure (Hare et al. 2010). This is true in other viviparous lizards (reviewed by Hare and Cree, 2010). It is our contention that these factors may at least partially explain why no inseminated females birthed live young rather than failure of the inseminating technique *per se*, and mitigation of these factors in future studies is recommended.

Nevertheless, should artificial insemination with fresh semen be successful, the next step is to examine fertility after insemination with short-term (liquid) stored sperm and ultimately with frozen-thawed (long-term stored) samples. The latter will require initial development of specialist cryoprotective media and confirmation of sperm functionality *in vitro* prior to testing it *in vivo*. Given that structural integrity of frozen-thawed sperm is likely to be compromised compared with fresh semen, it is possible that later inseminations may need to be considered, simply because frozen-thawed sperm may not survive 'stored' in the female reproductive tract for extended periods. If this approach is successful, then a tool for banking sperm from genetically valuable males could be realized (Holt et al. 2003).

WHAT IF WE CAN TEACH OLD TRICKS TO LIZARDS?

One of the immediate and urgent goals of the Grand and Otago Skink Recovery Plan is to secure both species in captivity (Norbury et al. 2006) and a Captive Management Plan has been prepared and implemented (Collen et al. 2009). Development of assisted breeding for McCann's Skink offers much promise as tools to assist this effort but the challenge will be effectively adapting these same techniques to threatened lizards. At the very least, this will increase our knowledge of their unique reproductive mechanisms. For example, semen collection and assessment could be used routinely to monitor when, or indeed if, male lizards are producing sperm during the breeding season. Artificial insemination could be used to unravel some of the complexities associated with sperm storage and competition in females and why sperm from some males and not others successfully sire offspring. Short-term (liquid) and indeed long-term (frozen) storage of sperm means that moving male gametes rather than animals from the wild or captivity is a realistic option for future breeding programs and for establishing sperm banks, and may reduce disease transmission risk between populations. The latter is particularly relevant given that ectoparasites are associated with pregnancy failure of McCann's Skink in captivity (Hare et al. 2010) and implicated in the cessation of breeding in captive Otago Skinks (Dennis Keall, pers. comm.).

Should artificial insemination prove to be successful, then captive breeding efforts and genetic management of animals bred for reintroduction to safe havens in the wild could be substantially improved and optimized. Otago Skinks have been bred successfully in captivity but efforts have met with little success in Grand Skinks (Norbury et al. 2006) until recently with a single birth recorded in January 2008 (Collen et al. 2009). Given that finite animals in the wild are available for translocation and the concomitant demand likely from captive breeding stocks, artificial insemination can help ramp up production of offspring in captivity to help increase the numbers of animals that will be required for restoration. The genetic distinctiveness of remnant existing wild populations of Grand and Otago Skinks has been determined (Berry and Gleeson 2005; Berry et al. 2005) and genetic analysis of captive animals recently has been completed (Dianne Gleeson, pers. comm.). The aim will be to breed animals to maximize genetic variability of offspring produced for reintroduction and artificial insemination offers a valuable tool in this regard, especially when selected animals cannot (due to geographical isolation) or do not (due to behavioural incompatibilities) mate. Not only will this maximize the efficiency of captive breeding effort but both founders and offspring can be monitored to ensure they are reproductively functional. Organized repositories of sperm banked from genetically valuable males will offer potentially useful support for managing and conserving Grand and Otago Skinks, as they do for other wildlife species (reviewed by Holt et al. 2003). The approach would be to map species-level genetic variation across the geographical range of each species (Allendorf and Luikart 2007; Palsboll et al. 2007), then optimise the number of individuals that require sampling to capture the genetic differences between populations. This would be contingent on fertility rates that could be achieved using frozen-thawed sperm (e.g., after artificial insemination). In this way maximal extant species variation could be safeguarded for generations to come long after donor males have died and offers a sensible and valuable back-up to *in situ* conservation programs.

Collectively, assisted breeding will inform on the reproductive mechanisms of threatened New Zealand lizards and in the longer-term provide useful tools to help produce high-quality conservation stocks for future restoration needs. As long as the information gained is integrated with that from other disciplines, in partnership with relevant stakeholders, end users, and management authorities, the most effective conservation outcomes will be realized.

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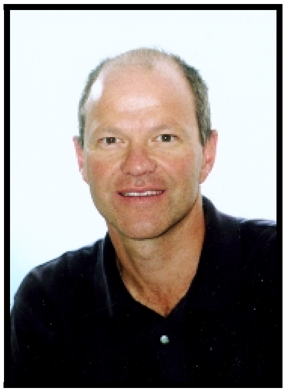
FRANK MOLINIA is a Programme Leader in the Biodiversity and Conservation team and Facility Manager of EcoGene™, a DNA-based diagnostic business at Landcare Research, Auckland. His research interests are in reproductive biology and specifically the development of reproductive technologies for conservation of threatened species and control of overabundant populations in Australasia. Other recent work with herpetofauna includes development of assays to non-invasively monitor the hormones of reproduction and stress from native frog species. (Photographed by Jennifer Teo).

TRENT BELL is a Herpetologist, who was formerly based at Landcare Research, New Zealand, and now runs a herpetological consultancy, EcoGecko Consultants. Trent is developing a national Web-database on New Zealand lizards. He investigates lizard taxonomy, and has described new species of skinks from the rugged mountainous terrain of the South Island. Trent's research interests also include developing effective sampling tools for highly cryptic species, developing active genetic management tools, and reintroduction projects of lizards. (Photographed by Dylan Van Winkel).



GRANT NORBURY is a wildlife ecologist at Landcare Research who specialises in predator-prey interactions and ecosystem dynamics in dryland environments. His recent interests focus on using native lizards as key functional components for assessing the effects of conservation management regimes. He is also chairman of a community conservation group that is restoring locally extinct lizard species in Central Otago. (Photograph courtesy of Landcare Research).

ALISON CREE is an Associate Professor at the University of Otago. She explores thermal effects on the reproduction of cool-climate reptiles, including applications to conservation. Her research involves several evolutionary lineages that currently or once inhabited southern New Zealand: viviparous geckos (*Hoplodactylus*, *Naultinus*), viviparous skinks (*Oligosoma*), and oviparous tuatara (*Sphenodon* sp.). (Photographed by Amanda Caldwell).

DIANNE GLEESON (left) is a research leader in the Biodiversity and Conservation team and Director of EcoGene™, a DNA-based diagnostic business at Landcare Research. Her research focuses on the development and application of genetic tools to conservation and pest management. Her herpetological research encompasses population genetics and phylogeography of a range of *Oligosoma* and *Cyclodina* species from New Zealand. (Photographed by Birgit Rhode).