LONGITUDINAL STUDIES ON TOOTH REPLACEMENT IN THE LEOPARD GECKO

by

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Abstract

The leopard gecko is an emerging reptilian model for the molecular basis of indefinite tooth replacement. Here we characterize the tooth replacement frequency and pattern of tooth loss in the normal adult gecko. We chose to perturb the system of tooth replacement by activating the Wingless signaling pathway (Wnt). Misregulation of Wnt leads to supernumerary teeth in mice and humans. We hypothesized by activating Wnt signaling with LiCl, tooth replacement frequency would increase. To measure the rate of tooth loss and replacement, weekly dental wax bites of 3 leopard geckos were taken over a 35-week period. The present/absent tooth positions were recorded. During the experimental period, the palate was injected bilaterally with NaCl (control) and then with LiCl. The geckos were to be biological replicates. Symmetry was analyzed with parametric tests (repeated measures ANOVA, Tukey's post-hoc), while time for emergence and total absent teeth per week were analyzed with non-parametric tests (Kruskal-Wallis ANOVA, Mann-Whitney U post-hoc and Bonferroni Correction). The average replacement frequency was 6-7 weeks and posterior-to-anterior waves of replacement were formed. Right to left symmetry between individual tooth positions was high (>80%) when all teeth were included but dropped to 50% when only absent teeth were included. Two animals were followed for 14 weeks after NaCl injections and 14 weeks after LiCl injections. NaCl did not affect the replacement dentition but LiCl delayed and disrupted the pattern of replacement. The phenotypes were more severe for one animal including 1) increased time before emergence, 2) increased total number of absent teeth per week, 3) a greater effect on anterior teeth and 4) disruption of symmetry. The most affected period began 7 weeks post LiCl injection. At the end of the study, in vitro CT scans of both animals revealed normal patterns of unerupted teeth however there was bone loss in one animal. Gecko tooth replacement is rapid enough to be useful for longitudinal studies. Between-animal variation is high when studying individual teeth therefore each animal should be used as its own control. Future work includes increasing the biological replicates and detailed molecular studies to confirm the effect of LiCl.

Preface

Leopard geckos were purchased from Triple R corns in Aldergrove, BC.

I took the wax bite impressions but Dr. Richman took some of them when I was away. Initial trials of different impression materials were carried out with the help of both Dr. Richman and Dr. John Whitlock.

Initial wax bite analysis and troubleshooting was carried out with help of postdoctoral fellow, Dr. Theresa Grieco. Inter-rater reliability scoring of the wax bites was done by Catherine Lee, a dental student at University of Pennsylvania in Philadelphia.

In-vivo and in-vitro µCT scans were completed at the UBC Centre for High-Throughput Phenogenomics with the help of the laboratory technician, John Schipilow, who ran the scans. I was responsible for sedating the geckos for the in-vivo scans. I created volumes and isosurfaces using Amira 3D software for life sciences (FEI Visualization Sciences Group).

I carried out the fixing, decalcification and processing into ethanol prior to embedding. Processing into wax was completed at UBC Dept. of Pathology. I positioned the specimens in wax molds, sectioned the blocks, put the sections on slides, stained the sections and took images of selected sections.

I assembled the figures for this thesis with the help of Dr. Richman. Statistical analysis was carried out with the help of Dr. Richman.

The animal work was completed with UBC Ethics Approval # A11-0352. This protocol was approved by the UBC Animal Ethics committee and is renewed annually.

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List of Abbreviations

APC – adenomatous polyposis coli protein
AXIN2 – axin related protein or axis inhibitor 2 protein
BIO – 6-bromoindirubin-3'-oxime
d – dentin
dl – dental lamina
EDTA – Ethylenediaminetetraacetic acid
GSK-3β – glycogen synthase kinase-3 beta
hb – healthy bone
la – labial surface
LiCl – Lithium Chloride
LiCl-1 – first 7 weeks following LiCl injection
LiCl-2 – second 7 weeks following LiCl injection
mt – midline tooth
NaCl – Sodium Chloride
NaCl-1 – first 7 weeks following NaCl injection
NaCl-2 – second 7 weeks following NaCl injection
p – palatal surface
pb – palatine bone
ppmx – palatal process of the maxilla
pt – pterygoid bone
s – premaxillary suture
sl – successional lamina
t1 – first generational tooth/erupted tooth
t2 – second generational tooth
t3 – third generational tooth
R - right
L - left
µCT – micro computed tomographic scan
UR – upper right
UL – upper left
ub – unhealthy bone
v - vomer
Wnt – Wingless Signaling pathway

Acknowledgements

I would first of all like to thank Dr. Joy Richman for accepting and allowing me to complete my master's research project in her lab. Her experience, dedication, help and excitement towards research were fundamental to my project's success.

Thank you to Dr. Greg Handrigan and Scott Holmes for their work in the lab on leopard geckos which my project is based on.

Thank you to Dr. John Whitlock for his help in the initial planning and troubleshooting stages of my project. John's experience in working with reptiles helped greatly in designing a feasible method to obtain wax bite impressions on the leopard geckos.

Thank you to Katherine Fu for all your guidance with histology and tissue processing.

Thank you to Dr. Theresa Grieco for your help with the initial analysis of the wax bite impressions data. You greatly helped in understanding what was initially a confusing set of data.

Thanks also to Catherine Lee for being willing to look at all those wax bites one more time. I am grateful to you for spending the hours necessary to validate my scoring!

Thank you to everyone in the lab for all your patience and support throughout my research project.

Thanks to Dr. Petros Papagerakis from the University of Michigan who looked at our problem with taking serial bites on geckos through a dentist's eyes. He wondered whether pink baseplate wax would work and he was right!

Thank you to my mother, Tuan and father, Kin for their continued support in all my endeavors and especially for vacuuming and cleaning my apartment.

Finally, thank you to my advisory committee: Dr. Cheryl Gregory-Evans and Dr. Virginia Diewert for your essential role in my research project and thesis.

Dedicated to my loving family. *You have always supported me throughout my life and throughout this whole experience.*

Chapter 1: Introduction

1.1 Mammalian tooth replacement and patterning

Replacement of dentition is common in many dentate vertebrates from fish to mammals. Reptiles such as snakes, lizards and crocodilians replace their teeth throughout their lifetime (polyphyodonty), but humans and other mammals are not able to renew their teeth indefinitely. Instead most mammals are only able to replace their teeth one time (diphyodonty) (Cobourne and Sharpe, 2010; Jernvall and Thesleff, 2012). Humans have two patterns of replacement within the 32 teeth that make up the dentition. The 20 teeth near the front of the mouth are replaced once however the posterior 3 molars are not replaced(Cobourne and Sharpe, 2010). The primary or deciduous dentition is replaced in the post-natal period by the secondary or permanent incisors, canines and premolars. In the molar region, the first molar forms at birth and the other 2 molars are added distally during childhood. Outside of primates, there are many variations in number of teeth that are replaced across mammalia. For example, the shrew (Järvinen et al., 2008; Yamanaka et al., 2007) and striped skunk do not replace their teeth at all (monophyodonty) (Mayer, 1969). Similarly, rodents do not replace their teeth but instead have evolved a continuously erupting incisor and non-replacing molars separated by a diastema. Thus there are no premolars in rodents (Jussila and Thesleff, 2012). The only two mammals that can replace certain teeth throughout life are the rock wallaby and the manatee (Jernvall and Thesleff, 2012). Thus it is hard to study tooth replacement in mammals so other models need to be found.

1.2 Non-mammalian tooth replacement

Non-mammalian models have been used for studies of tooth replacement due to increased opportunities to study the process during the life of the animal. The main models are fishes, reptiles and more recently sharks and rays. The bony fish such as the well-characterized zebrafish, cichlids and trout (Crucke and Huysseune, 2015; Fraser et al., 2008; Fraser et al., 2009; Huysseune, 2006; Huysseune and Witten, 2008; Vandervennet and Huysseune, 2005) can be used in a lab setting over long periods of time. One study added various chemicals to the water and affected tooth shape and replacement in cichlids (Fraser et al., 2013). Shark and ray tooth replacement has also recently been characterized since very little is known about their tooth replacement even though they are well-known to have many generations of teeth in development simultaneously (Smith et al., 2009; Underwood et al., 2015).

The reptile taxa consist of archosaurs (crocodilians and birds), squamates (snakes and lizards) and testudines (turtles). Birds and turtles have lost their teeth during the course of evolution (Lainoff et al., 2015). Their genomes no longer have functional copies of the genes coding for enamel proteins (Davit-Béal et al., 2009; Meredith et al., 2014; Shaffer et al., 2013). In the remaining dentate reptiles, most are polyphyodont except for certain agamid lizards such as bearded dragon (Cooper et al., 1970). As a point of reference, the leopard gecko is the one of the most basal lizards in the group Squamata (Vidal and Hedges, 2005). Tooth replacement in squamates has been studied in the past by our lab (Buchtova et al., 2013; Handrigan et al., 2010; Handrigan and Richman, 2010a, b) and others (Buchtova et al., 2013; Gaete and Tucker, 2013; Vonk et al., 2008; Zahradnicek and Horacek, 2008; Zahradnicek et al., 2008). Tooth replacement patterns have also been previously studied longitudinally in green iguanas (Kline and Cullum, 1984; Kline and Cullum, 1985), lizards,(Berkovitz, 2000; Cooper, 1964; Edmund, 1960), slow worm (Cooper, 1966) and alligators (Edmund, 1969; Osborn, 1998; Westergaard and Ferguson, 1987, 1990). Both fish and reptiles are suitable models however the reptile is more closely related to mammals and has very similar stages of tooth development. The fish and shark fold their epithelium to generate the next tooth generation which is different than in mammals. Thus, in addition to being unavailable to us, fish and sharks are less suitable models for our purposes.

1.3 Individual tooth development

The dental lamina, which surrounds the oral cavity, is present throughout the lifetime of the reptile. The successional lamina extends to the lingual beyond the terminal tooth in the family (Figure 1.1). For exfoliation to occur, the following tooth is fully mineralized and resorbs the majority of the erupted tooth. At the same time, 2-3 more teeth in the tooth family may be in development below the oral mucosa (Figure 1.3A) (Handrigan and Richman, 2011).

Figure 1.1 Tooth development in reptiles

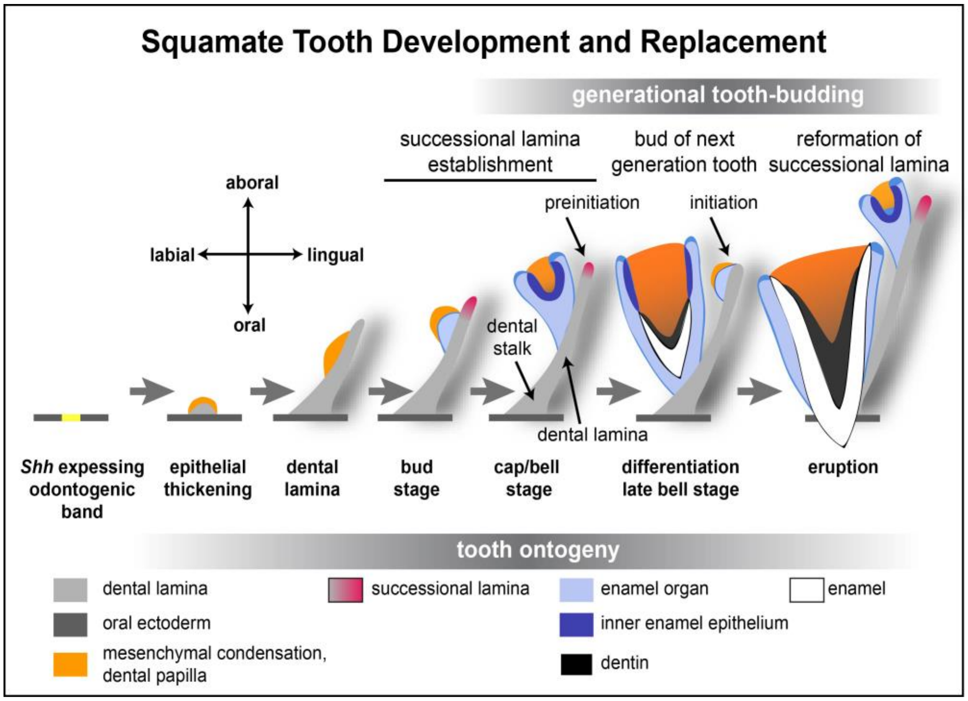

Fig. 1.1 - Schematic of tooth development in reptiles. Adapted from (Richman, 2013).

Squamate dentition develops similar to dental development in humans. Teeth initiate from the dental lamina through thickening/invagination of a band of oral epithelium. This delineates the location of where the tooth will develop in the arch. The dental lamina forms as this ingrowth of epithelial cells and interacts with the underlying mesenchyme. Further cell proliferation occurs and localized swellings develop on the labial side of the dental lamina which will form the tooth germs. Each tooth bud forms an enamel organ that then progresses to the cap stage and the bell stage (Jussila and Thesleff, 2012; Laurikkala et al., 2003; Thesleff et al., 2001). The enamel organ consists of an inner and outer enamel epithelium as well as a stellate reticulum between them. Mammals have an additional layer called the stratum intermedium separating the stellate reticulum from the inner enamel epithelium. A similar layer is not present in squamates (Handrigan and Richman, 2011) and crocodilians (Wu et al., 2013). Mammals also have clearly defined enamel knots that determine the number of cusps (Jernvall and Thesleff, 2012). Since most reptiles have simple conical teeth, enamel knots are not present (Handrigan and Richman, 2011).

Morphodifferentiation (increase in size and development of shape) and histodifferentiation occurs as time progresses. Histodifferentiation occurs when the odontoblasts developed from the mesenchymal-derived dental papilla begin to deposit dentin. Immediately after the first layer of dentin is secreted, the inner enamel epithelium forms polarized ameloblasts which begin depositing enamel. In reptiles and in the primary dentition of diphyodont mammals the outer enamel epithelium of the enamel organ forms a successional lamina. Once the successional lamina reaches a certain length, another tooth bud begins development. In mammals the successional lamina only forms once and degenerates after the permanent dentition is formed (Buchtova et al., 2013; Richman, 2013).

The cervical loops may carry on forming Hertwig's epithelial root sheath which defines the number and shape of the roots (Diekwisch et al., 2006). All reptiles, except crocodilians have ankylosed teeth in which there is no cementum and the tooth dentin merges directly with the bone (Diekwisch, 2001). In lizards and snakes, the majority of animals have a pleurodont tooth attachment. This means that the labial side of the tooth is fused to a slanting bone ridge while the lingual side is not covered by bone. In alligators and crocodiles, teeth are set in a bony socket with both the buccal and labial sides of the root covered by bone. This attachment of tooth to bone via a periodontal ligament is termed thecodonty and is the type of tooth attachment present in mammals (Berkovitz, 2000).

1.4 Regulation of tooth replacement in reptiles

Tooth replacement in squamates also appears to be an event which happens as a scheduled event rather than being due to breakage or destruction of the tooth (Berkovitz, 2000; Edmund, 1969). It is therefore not necessary to extract teeth to initiate the process of tooth replacement. The proof of this statement is scant since Edmund did not publish the data that demonstrated extractions had no effect on the speed or pattern of tooth replacement. We know from a student that works at the Royal Ontario Museum, where Edmund was the curator, that he did extract teeth from the green iguana (personal communication, Kirsten Brink, Royal Ontario Museum). It will be interesting to revisit the radiographic data collected on these iguanas to determine more precisely the effect on the unerupted teeth as well as emergence into the oral cavity.

Tooth replacement/exfoliation patterns, as previously studied in the green iguana (Kline and Cullum, 1984; Kline and Cullum, 1985), common lizard (Berkovitz, 2000; Cooper, 1964;

Edmund, 1960), slow worm (Cooper, 1966) and alligators (Edmund, 1969; Osborn, 1998; Westergaard and Ferguson, 1987, 1990), followed a posterior to anterior exfoliation pattern when alternate tooth positions were connected (Figure. 1.2). In a graphical representation of the teeth in the arch, the next tooth to exfoliate is usually one tooth position away rather than the tooth directly adjacent to/in front of that tooth.

Figure 1.2 Tooth replacement/exfoliation pattern as shown previously in the green iguana

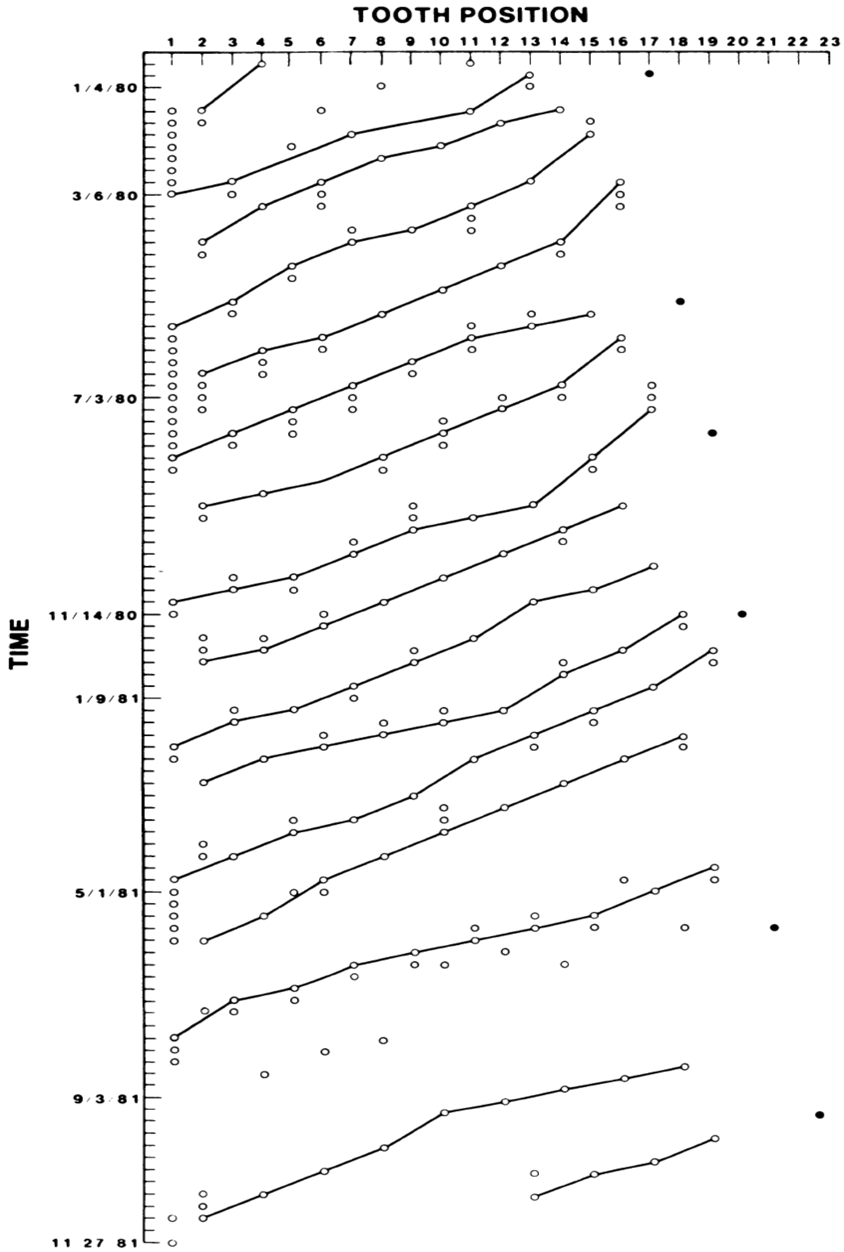

Fig. 1.2 – Graphical representation of tooth replacement/exfoliation patterns in the green iguana. Blank spaces are the present teeth in the quadrant. Open circles are the absent teeth. Diagonal lines are connecting every other absent tooth in the posterior to anterior pattern. Taken from (Kline and Cullum, 1984).

Each individual tooth took up to 3 months to replace in the green iguana (Kline and Cullum, 1984; Kline and Cullum, 1985), approximately 7-8 weeks in the slow worm(Cooper, 1966), up to 38 weeks in the common lizard (Berkovitz, 2000; Cooper, 1964) and up to 18 months to replace in the alligator (Kline and Cullum, 1984; Kline and Cullum, 1985; Osborn, 1998; Westergaard and Ferguson, 1987, 1990). The green iguana, common lizard, and slow worm tooth replacement/exfoliation patterns were recorded with baseplate wax bites while the alligator's tooth replacement patterns were recorded with serial radiographs. The term "waves of tooth replacement" was used by Edmund to describe the pattern. He did a large comparative study of modern and fossil lizard specimens at the Royal Ontario Museum (Edmund, 1960; Edmund, 1969).

The unerupted teeth in the jaw are surprisingly not all at the same stage of development. In other words the tooth that is directly under the erupted tooth may range in development from being nearly ready to come in to an immature dental bud with no signs of enamel or dentin. Or in other words, two unerupted teeth of similar stages of development will belong to different tooth families. While this may seem disorganized, the neighbouring unerupted teeth form a striking pattern when viewed down the entire jaw (Figure. 1.3B). When arranged from most to least mature, the unerupted teeth form diagonal lines relative to the dental arch. The term Zahnreihen or "tooth row" was coined by Woerdeman to describe this diagonal arrangement and the term was adopted by Edmund in 1960 (Edmund, 1960; Woerdeman, 1921). It is important to recognize that in order to see these diagonal lines, it is necessary to use radiographs or histology.

Figure 1.3 Tooth families and Zahnreihen as seen by μCT scan

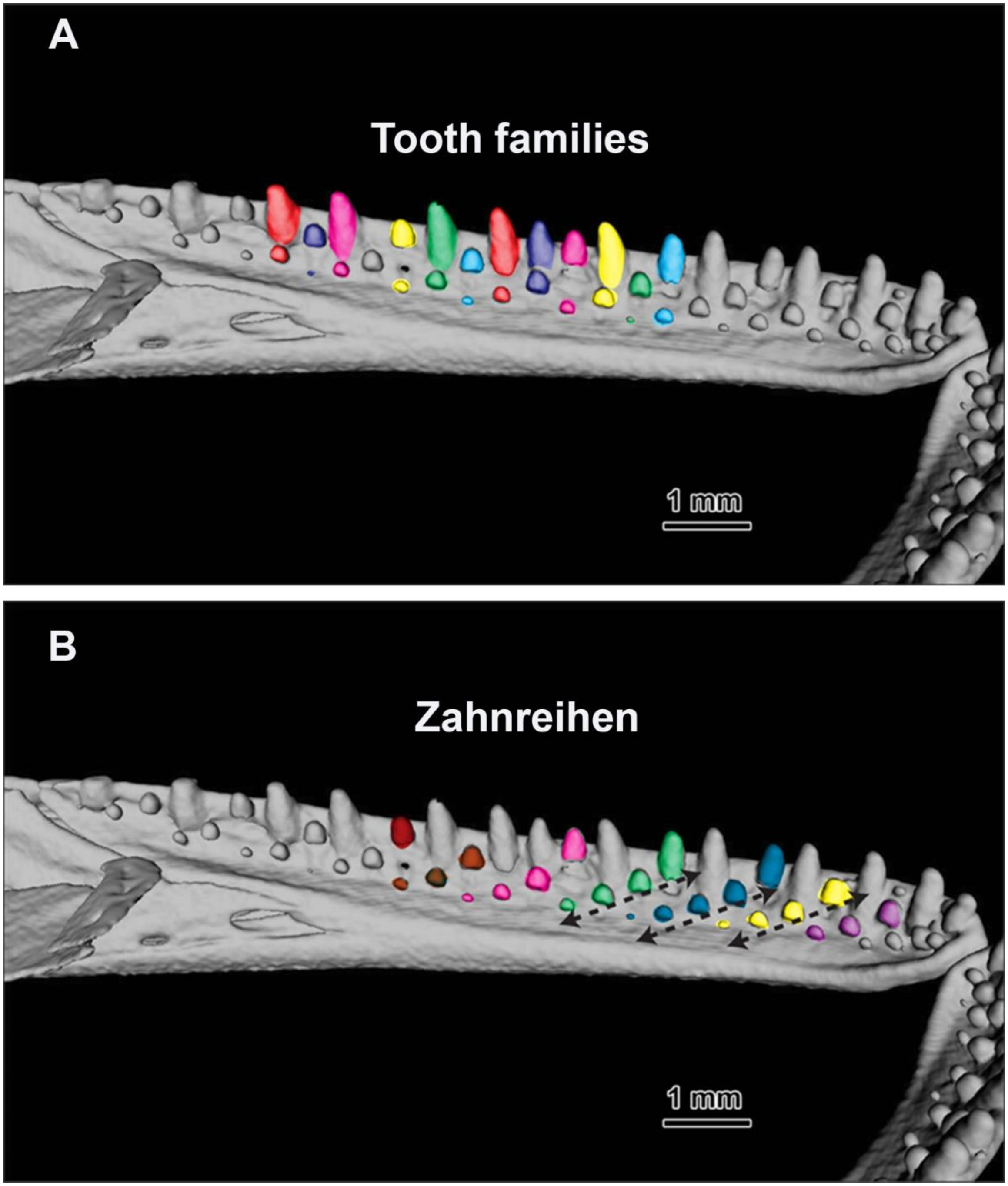

Fig. 1.3 - μCT scan of the mandible of a young leopard gecko showing tooth families by color (**A**) and Zahnreihen by color (**B**). Taken recently in the Richman lab as part of a separate research project of Dr. Theresa Grieco.

1.5 Reason for using the leopard gecko for investigations

Various other reptilian models have been previously used for studying tooth development and replacement. However we wanted to focus on in vivo replacement in post hatching animals which limited our options. Animals that have been used include the green iguana (Kline and Cullum, 1984; Kline and Cullum, 1985) alligator (Westergaard and Ferguson, 1987; Wu et al., 2013) (Edmund, 1962). There are obvious safety concerns with carrying out tooth studies on alligators. Both alligator and iguana take a large amount of space and resources to rear in a lab setting. Also both animals take a long time for teeth to be replaced We were therefore searching for a different reptile model that had the same type of tooth replacement pattern noted by Edmund on alligators but with more rapid turnover and ease of handling for tooth replacement manipulation experiments. We identified the leopard gecko (*Eublepharis macularius*) as a potential model but found that this animal had not previously been investigated in this capacity.

Our lab has previously published articles on embryonic and post-hatching adult geckos and we are developing the leopard gecko to be a model organism for all forms of tooth replacement research (Handrigan et al., 2010; Handrigan and Richman, 2011). A staging table has been developed for the prehatching leopard gecko (Wise et al., 2009).

1.6 Canonical Wnt signaling as a candidate signal involved in tooth turn-over

The molecular reasons for indefinite tooth replacement are the next big question we wanted to address. Prime candidate signals that could be involved in tooth turn-over include those in the canonical Wingless signaling pathway (Wnt). When this pathway is active, β catenin is stabilized, accumulates in the nucleus and triggers a cell response. Mouse studies have shown that increased canonical Wnt signaling in the oral epithelium leads to a large increase in the number of teeth (Järvinen et al., 2006; Wang et al., 2009). In humans, mutations in pathway mediators AXIN2 (Lammi et al., 2004) or APC (Half et al., 2009) lead to tooth agenesis or supernumerary teeth respectively. Mutations in APC cause Gardener's syndrome (Groden et al., 1991) , which includes formation of many supernumerary teeth. Studies in our lab looking at the activation of the canonical Wnt pathway have shown increase in proliferation of cells in the dental lamina in snakes (Handrigan and Richman, 2010b) and geckos (Handrigan et al., 2010). This suggests the possible involvement of the canonical Wnt signaling pathway in the replacement of teeth in reptiles.

1.7 LiCl is an activator of the canonical Wnt pathway

LiCl has been studied in the past to be an activator of the canonical Wnt signaling pathway. The effect of LiCl on the canonical Wnt pathway was initially proposed by researchers studying the mechanisms by which Lithium treats bipolar disorder (Klein and Melton, 1996) and was further studied in Drosophila and Xenopus (Hedgepeth et al., 1997). LiCl activates the canonical Wnt signaling pathway through inhibition of the GSK-3β, which is a negative regulator of the Wnt signaling pathway. More recent studies show that LiCl can overactivate the canonical Wnt signaling pathway causing inhibition of palatal fusion and osteogenic differentiation in mouse palatal shelves in vitro (Meng et al., 2015). In addition, LiCl has been recently tested successfully in vitro on human first molars as a potential pulp capping material. LiCl applied to coronally amputated pulp chambers caused dentin regeneration/bridge formation through the activation of the canonical Wnt signaling pathway which is a regulator of dentin sialo phosphoprotein (important in dentin formation) (Ishimoto et al., 2015). In our lab, LiCl was used in python snake organ cultures to activate the canonical Wnt pathway which resulted in increase of *Lef1* and *Axin2* expression verifying the gain-of-function effect of LiCl on the canonical Wnt pathway (Handrigan and Richman, 2010b). LiCl was therefore chosen as the activator we decided to use for our study on tooth replacement.

1.8 Aims and goals of my study

The first goal of my study is to develop a different reptile model which has the same type of tooth replacement noted previously but with more rapid turnover. Secondly, I would like to characterize the normal tooth replacement pattern in the leopard gecko (*Eubelpharis macularius)*. This has not been studied in the past and is essential to developing a model for tooth replacement studies. Thirdly, I hope to determine the clinical effect of LiCl injections on tooth replacement over time. The hypotheses are that 1) the leopard gecko has posterior to anterior waves of tooth replacement similar to those of other reptiles along with similar Zahnreihen but with faster tooth turnover, and that 2) stimulating the Wnt pathway with LiCl will increase the rate of and/or change the waves of tooth replacement.

The novelty of my work is that while tooth development is a topic of study in many vertebrates, the underlying mechanisms that regulate renewal of teeth are largely unknown. The emphasis in most studies is instead on individual tooth morphogenesis rather the budding of generational teeth. Yet, it is very important to find an animal in which we can tease apart the molecules that can trigger tooth formation from existing dental tissues. The significance of this research on geckos is that one day it will lead to treatments for the replacement and/or regeneration of lost, unrestorable or missing teeth in humans.

Chapter 2: Materials and Methods

2.1 Leopard gecko husbandry

Adult leopard geckos were obtained from a local breeder (Triple R Corns, Aldergrove, BC). They were kept in terraria which were heated by heat pads attached to half the bottom surface. This allowed the geckos to regulate their own temperature. "Hide" containers were placed in each terrarium to provide the animal with a dark place to hide from external stimuli which may stress the animal. Damp paper towels were placed in the containers to aid the animals in shedding of their skin. Water dishes were replenished and cleaned daily. Mealworms were gut-loaded for two days with a mixture of dried dog food, skim milk powder, oats, a reptile vitamin powder blend, and a carrot (as a water source). The mealworms were fed to the geckos every 1-2 days. Records were taken daily for tracking the cleaning of the terraria, feeding and water changes. Animals were also weighed each week to ensure the continued health of the geckos throughout the experimental period.

2.2 Serial wax bite impressions and counting

Wax bite impression of the upper jaw were to be taken with dental baseplate wax every week on three leopard geckos for a total of 35 weeks. While the geckos were awake, the lower jaw of the gecko was touched gently which caused the gecko to open quite wide. With the gecko's mouth wide open, a small piece of baseplate wax was placed in between the gecko's upper and lower jaws. Once the baseplate wax reached the corner of the gecko's mouth, the gecko instinctively closed down on the wax bite resulting in a clear imprint of the present/absent teeth in the upper/lower arch (Figure 2.1 A, B, C). This allowed for a relatively safe and simple way to record tooth exfoliation of the leopard gecko's teeth over time similar to the method used on slow worm, common lizard and green iguanas previously (Cooper, 1964; Cooper, 1966; Kline and Cullum, 1984; Kline and Cullum, 1985).

These wax bite impressions were then imaged with a Leica M125 stereo microscope at 8X magnification to visualize the present and absent tooth imprints. The bites were scored for the absence/presence of each tooth in the arch/row.

Figure 2.1 Wax bite impressions and palatal shelf injections in the leopard gecko

Fig. 2.1 – Procedure of wax bite impression: (**A**) opening of the gecko's mouth through touching the gecko's lower jaw, (**B**) placement of the baseplate wax while mouth is open, (**C**) imprint of teeth into wax when gecko bites together, (**D**) injection of the palatal shelves with an insulin syringe and pipet tip as a bite block under Isoflurane anesthesia**.**

2.3 Collection and scoring of wax bites

Upper wax bites were taken weekly for 3 geckos, LG59, 60 and 100 (LG – leopard gecko, the number refers the individual animal within the series of geckos purchased in the lab). The ages were not known but they were similar weights and appeared to be mature geckos. LG100 was acquired approximately 4 months after LG59 and LG60. Unfortunately, we were not able to reliably capture the mandibular posterior teeth (Figure 2.2A,A'). Likely reasons for the absence of mandibular tooth indents are either that the tongue partially blocked the imprint of the bite on the baseplate wax or that the gecko did not bite down hard enough to make an imprint. Another region that was not always present in the wax bites was the posterior of the upper jaw. Typically, an imprint of the last five teeth in the arch was missing in many of the bites. Since teeth seemed to be added posteriorly during the 8 months of data collection, the total number in each animal was slightly different. This addition of teeth as the geckos continue to grow has been previously described (Thorpe, 1983). To make the data more consistent, we focused our analysis on teeth 1-35 of the maxillary quadrants. Partially erupted teeth were visible in the wax bites (Figure 2.2C').

The foundation of our analysis rests on being able to identify specific teeth according to their position in the jaw. Since all teeth are identical in morphology (homodont), it was necessary to use anatomical landmarks. The midline tooth was initially determined through comparison of the number of teeth on the right and left and confirming they were equal. However, due to the variable number of teeth imprinted in the bites (especially since teeth were added posteriorly and the most posterior teeth often missing), the tooth number on both left and right sides were not always equal. It was essential to come up with a more reliable method of determining the midline.

Since the gecko has an upper midline tooth interdigitated in the mandibular symphyseal gap, we used the lower anterior midline gap as an indicator of the midline of the upper arch. Here we took advantage of the fact that the lower anterior teeth and midline gap was always visible on the wax bite. The location of the midline gap was marked/scored on the lower side of the wax bite and transferred to the opposite side to determine the true midline tooth location in the upper arch (Figure 2.2 B,C).

To ensure that the scoring of absent teeth were as accurate as possible, the wax bites were scored 4 times, twice by the principal investigator and twice by an independent rater.

Figure 2.2 Wax Bite impressions and Scoring

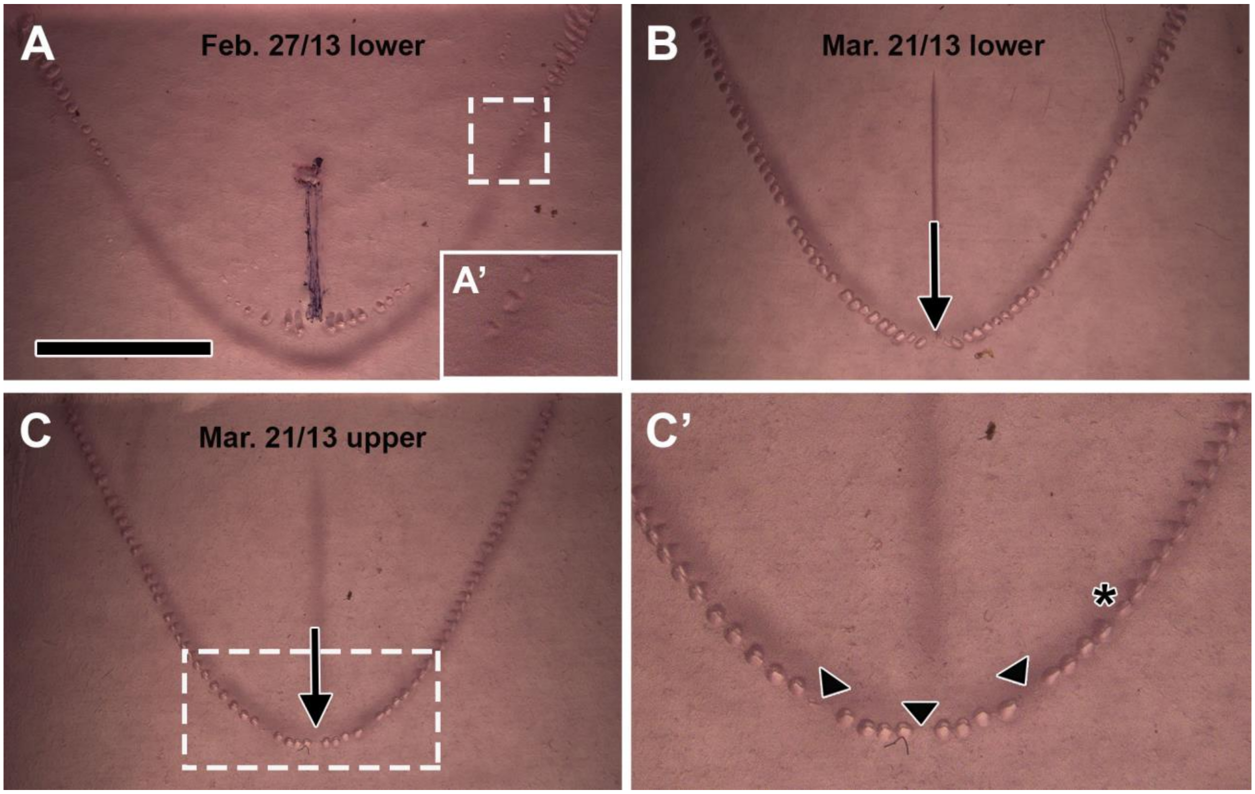

Fig. 2.2 – Multiple wax bites taken at different times illustrate the collection and scoring of the present/absent teeth. (**A**) Lower wax bite impression with midline marked and with lack of posterior bite imprints (**A'**). (**B**) Lower wax bite indicating the lower symphseal gap indicating where the upper midline tooth interdigitates (arrow). (**C**) Upper wax bite indicating the upper midline tooth position as transferred from the lower (arrow). (**C'**) Zoomed in view indicating absent (arrowheads), and partially erupted scored teeth (asterisk). Scale bar for A,B,C = 5mm; Scale bar for A',C' = 2.5mm.

2.4 Oral NaCl and LiCl injection

Oral administration of 0.1mg/kg body weight Meloxicam (a common veterinary non-steroidal analgesic) was given to the leopard geckos approximately 12 hours prior to treatment in order to prepare them for the injections. To carry out the injections, the animal was first placed under deep sedation using 5% Isoflurane/oxygen. Throughout the procedure, the animal could be observed to be breathing on its own. The mouth of the animal was then propped open using a pipet tip and local anesthetic consisting of 50μL of 0.5% Lidocaine with 1:250,000 epinephrine was injected into the palatal shelves on both sides (Figure 2.1D). The reasoning for this initial injection was to anesthetize the area prior to LiCl injections and to cause local vasoconstriction to keep the solutions at the injection site for a longer time. Using dental surgical loupes 2.5x magnification, either 100 μL of 1M NaCl (control) or 1M LiCl was injected bilaterally to the base of the teeth in the palatal shelves using an insulin syringe. To ensure the injections were correctly positioned, we watched for the palatal shelves to inflate with solution. This indicated the solution was retained in the tissue. If the needle went through the palatal shelves, the solution could be observed being ejected to the mouth and needed to be pulled back slightly to be retained within the tissue. A concentration of 1M LiCl was used since it has been previously shown to have an effect on proliferation in the dental lamina in vivo by a previous graduate student (Holmes, 2013). In addition this same concentration was used in vitro on snake organ cultures (Handrigan and Richman, 2010b). The animal was then given oxygen and given time to awake. During this time, the geckos were monitored for continued breathing and increased alertness/responsiveness. The geckos were also checked the following day to ensure there were no lasting effects. These injections were delivered every other day for a total of 3 injections over a period of 1 week. The reasoning behind this was to give the area continued exposure over the week since LiCl is a very short-acting molecule. The geckos were then followed with wax bite records each week for a period of 14 weeks after each injection. For analysis purposes each 14 week period was divided into two 7 week blocks, NaCl-1, NaCl-2, LiCl-1, LiCl-2.

2.5 Micro-CT scans (in-vivo with sedation and in-vitro)

We conducted µCT scans in order to help to visualize the possible presence of Zahnreihen in the leopard gecko. An in-vivo µCT scan was carried out while we were still taking wax bite impressions followed by an in-vitro µCT scan after euthanasia.

In order to do the in-vivo µCT scan, the geckos had to be sedated and non-mobile throughout the scan. Due to the difficulty maintaining a deep plane of anesthesia in the CT scanner, a different drug, Alfaxalone was used. Alfaxalone is a neurosteroid which acts on the GABA receptors in the brain producing sedative effects similar to Propofol. Previous studies of intramuscular Alfaxalone in reptiles showed that it is a relatively fast and reliable way to obtain short sedation or anesthesia in healthy green iguanas (Bertelsen and Sauer, 2011). We therefore gave the geckos 30 mg/kg body weight of 10mg/mL Alfaxalone (maximum volume 100µL) injected into the sternocleidomastoid muscle to allow for the in-vivo CT scans to be completed without the geckos waking and moving around. A maximum resolution of 50µm was possible for in vivo scans (TriFoil eXplore CT 120), considering the acceptable radiation dose to the animal (scan time 4 minutes, 175 mGray). After the scan was complete the gecko was given oxygen and monitored for breathing and responsiveness. In vitro scans were carried out at a resolution of 20µm (Scanco Medical µCT-100).

The CT scans were viewed and 3D reconstructions were made using the Amira 3D software for life sciences (FEI Visualization Sciences Group). The µCT scanners are located at the UBC Centre for High-Throughput Phenogenomics.

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2.6 Statistical analysis of symmetry, tooth absent number per week and eruption time of the replacement tooth following exfoliation

All statistical analysis was carried out with Statistica v.7 (Statsoft) software.

For symmetry analysis, we analyzed the data in 2 ways. First, the number of present and absent teeth that were identical on both the left and right sides was determined. The percentage symmetry for each week was calculated by dividing the identical teeth by the total number of teeth in each maxillary quadrant. In the second analysis, only absent teeth were included. The dependent variable was the % symmetry between the right and left sides and the independent variables were the control period, the 2 NaCl and the 2 LiCl periods. The control period was compared to both the NaCl and LiCl periods for each animal. Due to the parametric nature of the data (continuous percentages, normally distributed data (Shapiro Wilk W test) and homogeneity of variance (Levene Test)) and comparison because our within animal analysis was not entirely independent, the repeated measures ANOVA was performed followed by the Tukey's post-hoc testing (if required) to determine differences between groups. P values less than 0.05 were considered statistically significant.

To determine the number of teeth absent per week, we counted the number of missing teeth each week and to determine the length of time for the replacement tooth following exfoliation of the previous tooth, we counted the number of weeks a tooth was absent before being replaced by another tooth The control period was compared to the NaCl and LiCl periods.

Due to the non-parametric (discontinuous data, non-normally distributed data (Shapiro Wilk W test)) nature of both these sets of data and comparison among multiple samples, the Kruskal-Wallis test was performed followed by the Mann Whitney U post-hoc testing to determine differences between groups. P values less than 0.05 were considered statistically significant. The Bonferroni multiple testing correction ($p>0.05$/number of comparisons) was also applied when the number of comparisons increased above 2.

To determine regional effects of palate injections, we analyzed the arch in 5 segments of 7 teeth. We chose 7 teeth because tooth positions 1-7 are contained within the premaxilla and the number is evenly divisible into 35.

2.7 Tissue preparation for histology

Adult gecko jaws were initially dissected. They were then placed into Bouin's fixative for 24 hours, followed by decalcification in Morse solution at room temperature for 48 hours (Nakatomi et al., 2006). Morse solution is composed of 10% w/v sodium citrate and 22.5% v/v formic acid and if after the 48 hours, the bone was not soft enough to be probed with a pin, it requires a longer period of decalcification. The Bouin fixed gecko jaws were then washed in multiple changes of 70% ethanol to get rid of the remaining fixative prior to wax processing.

2.8 Histologic staining with Alcian blue and Picrosirus red

The gecko jaw sections were processed into paraffin wax and were cut coronally into 7µm sections. These sections were mounted onto Superfrost Plus slides (Fisherbrand) and baked at 65°C for 8-12 hours. Excess wax was removed from the slides with two 20 minute xylene baths. The sections of tissue were then rehydrated with a graded series of alcohol baths. Tissues were washed in double distilled water prior to being submerged in 1% Alcian blue stain for 30 minutes at room temperature. Tissues were then placed in 1% acetic acid, washed with double distilled water again, and then submerged in Picrosirius red for 1 hour. Tissues were again placed in 1% acetic acid, washed with double distilled water and then dehydrated with a graded series of alcohol baths. The sections of tissues were then placed back into a xylene bath prior to mounting the slides with cover slips with Consul Mount (Thermo Scientific). After allowing a couple days for the mounting medium to set, images were taken with Zeiss Axioskop at various magnifications. Histologic sections allowed us to view the basic anatomy and bone development around tooth families.

Chapter 3: Results

3.1 Typical dental morphology and arrangement in leopard geckos

Of the three geckos studied in our experiment, there were on average 40 teeth per quadrant. The teeth of the geckos themselves are homodont in an ovoid arch form and when viewed from the occlusal, the crown is conical (Figure 3.1A,A'). There is a mild cingulum structure which is difficult to see clearly on the lingual half of the tooth. Thus gecko teeth have 2 cusps as previously described (Handrigan and Richman, 2011). When viewed from the buccal/lingual, the anterior surface is inclined and curved with the incisal surface relatively flat. The enamel is relatively translucent and thin. The mesial to distal width of the tooth is on average 250 µm (Figure 3.1A',B). The in-vitro CT scans reveal the pleurodont tooth attachment that is typical of most lizards and snakes. The buccal surface of the root is fused to the bone while the lingual part of the tooth sits on top of the bone. In addition there are no roots in pleurodont dentition, therefore there is no periodontal ligament or tooth socket. Gecko teeth are ankylosed to the bone and only become loose when the next generation tooth that is always lingual to the erupted tooth, begins moving buccally, thereby resorbing the bone-tooth attachment (Figure 3.1A'). The unerupted replacement teeth are visible in various stages of development on the CT scan and skeletal preparations because they lie external to the bone (Figure 3.1A,A',C). The gecko dentition also has with a single upper midline tooth (Figure 3.1D). The midline maxillary tooth appears to interdigitate with a mandibular symphyseal gap between the dentary bones which are not fused in geckos.

Figure 3.1 Dental morphology and arrangement of teeth in the leopard gecko

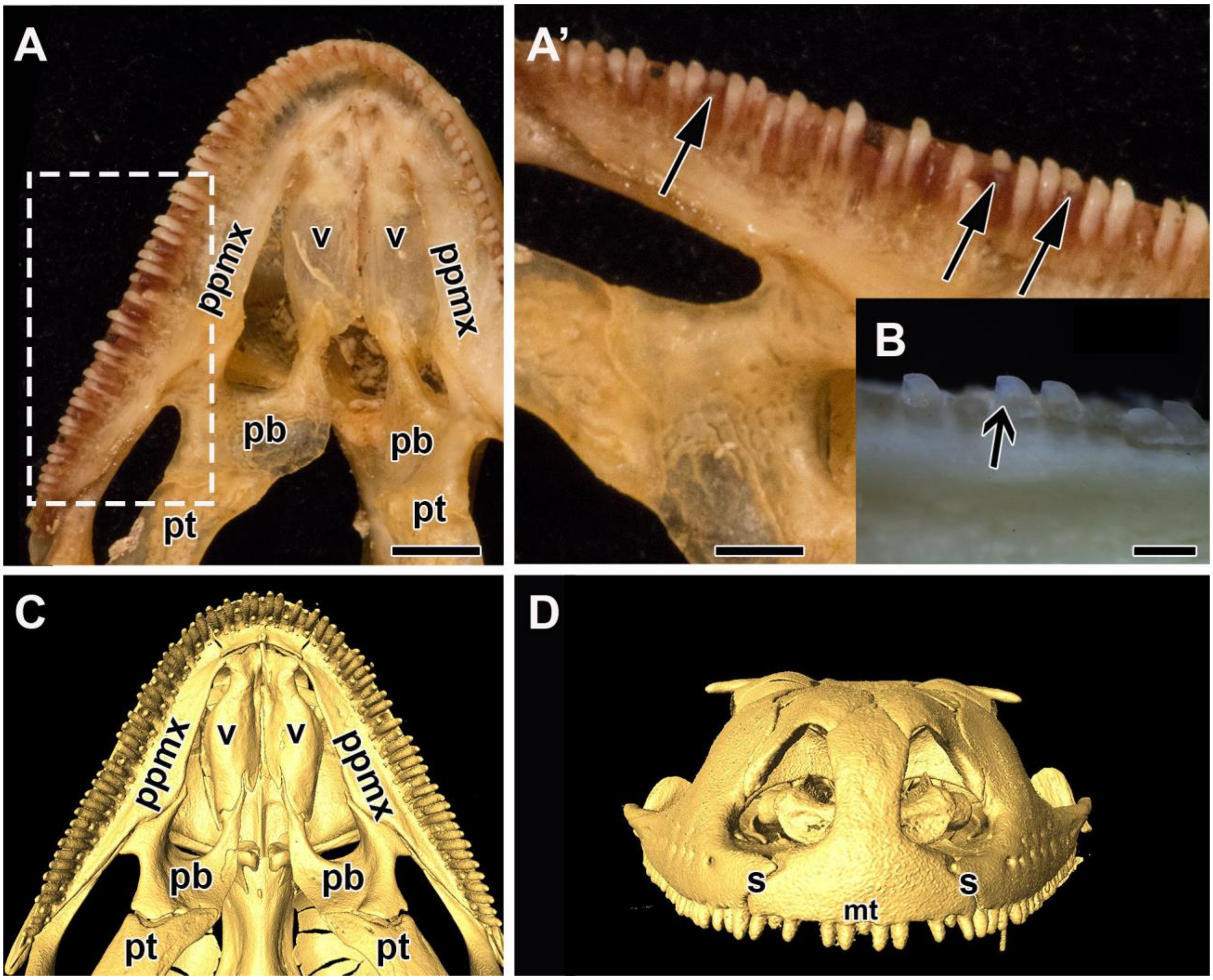

Fig. 3.1 – View of the dentition and upper jaw of the leopard gecko. (**A**) Preserved skeletal specimen of the leopard gecko, photos taken by Dr. Richman at the Royal Ontario Museum. The resorption of the erupted teeth by the developing tooth is indicated by the palatal view of the upper right side of the arch (arrows in **A'**). (**B**) Wet specimen of one of the geckos in the experiment as viewed from the lingual. Translucency of the enamel can be visualized (tailed arrow). (**C, D**) In-vitro CT scans of untreated young leopard geckos courtesy of Dr. Theresa Grieco. Key: mt, midline tooth; pb, palatine bones; ppmx, palatal processes of maxilla; pt, pterygoid bones; s, premaxillary sutures; v, vomer. Scale bar in A = 2mm; Scale bar in A' = 1mm; Scale bar in C = 500µm.

3.2 Intra and inter-rater reliability of wax bite impression tooth counts

Both intra and inter-rater reliability were calculated which showed quite high reliability of 99% intra-rater and 98% inter-rater reliability (Table 3.1). Any scored values which were different were discussed by the raters and tooth present/absent/partially erupted score was mutually agreed upon. The scored values which differed were due to disagreement in single teeth being present/absent/partially erupted rather than shifts in the midline. Therefore the scoring of the midline was consistent.

Table 3.1 Intra-rater and Inter-rater Reliability of Wax Bite Scoring

	Rater 1 Intra-Rater Reliability	**Rater 2 Intra-Rater Reliability**	**Inter-Rater Reliability**
Animal/Side	**Non-Matching Counts**	**Non-Matching Counts**	**Non-Matching Counts**
LG 59L	10	8	16
LG59R	11	9	19
LG60L	13	15	22
LG60R	13	13	19
LG100L	5	4	7
LG100R	3	3	6
Total Non-Matching Counts	55	52	89
Total Matching Counts	5489	5492	5455
Total Counts	5544	5544	5544
Reliability %	99.01%	99.06%	98.39%

3.3 Optimal periodicity for wax bites determined by the time for tooth eruption after exfoliation

First we wanted to determine the ideal periodicity for taking wax bites. This timing was important because we wanted to capture all tooth loss events. If we sampled too infrequently, we could miss out many data points. If we sampled too frequently we would have redundant data that did not add to the story. We compared taking bites every third day compared to weekly bites for two of the animals, LG59 and LG60. The bites taken every 3rd day gave similar results to weekly bites. In other words, the same teeth that were absent after 3 days were still absent at 7 days (Figure 3.2A-D). Also, histograms comparing time for tooth eruption after exfoliation showed that 6 days was the most common amount of time required and with an average of 6.9 days for both animals (Figure 3.2E,F). We therefore decided to continue with weekly wax bites for the remainder of the study since very few, if any, tooth replacement events would be accidentally excluded.

Figure 3.2 Wax bites taken every 3 days

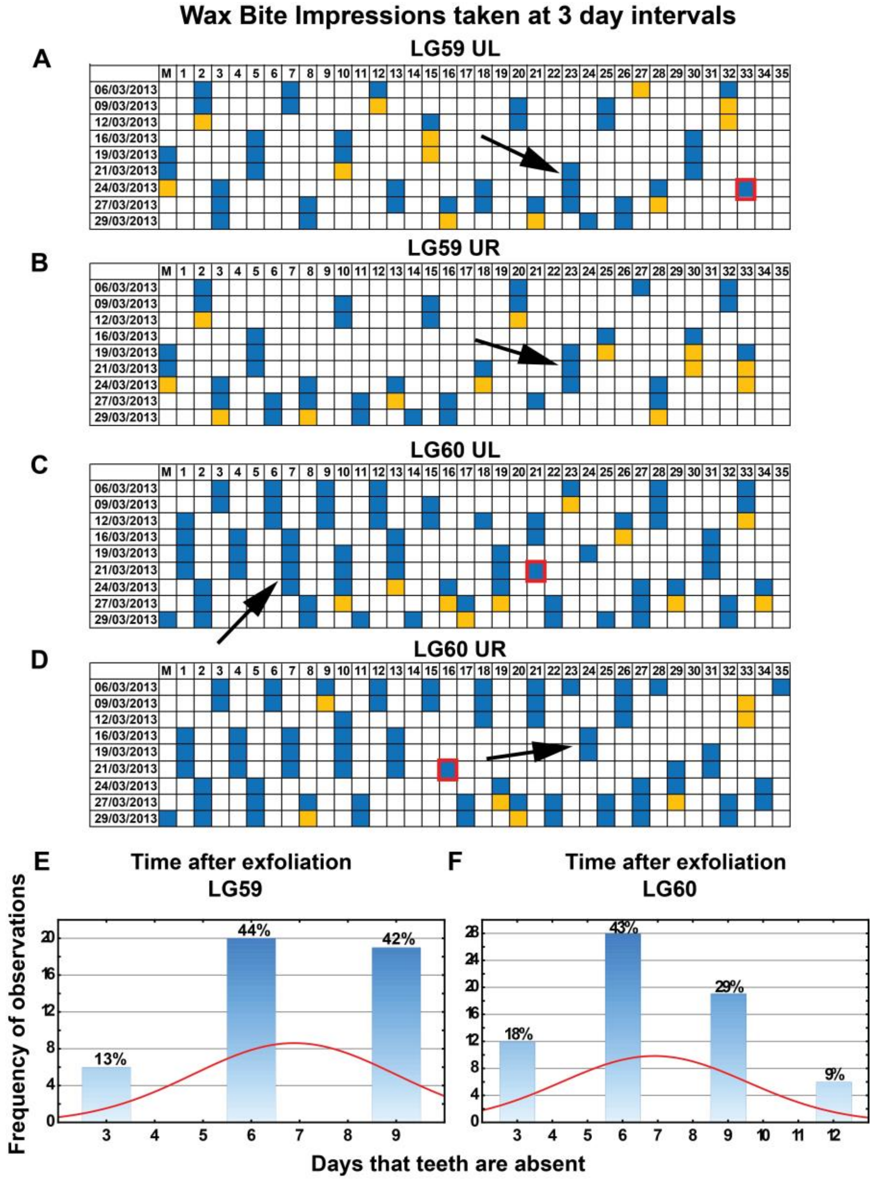

Fig. 3.2 – Records of wax bites taken at 3 day intervals to test for the best time frame to take wax bite impressions on the leopard gecko. (**A-D**) Illustration shows teeth absent and present over a one month period with wax bites taken every 3 days on LG59 and LG60 upper left (UL) and upper right (UR). Rows indicate impression dates. Columns indicate tooth positions. Blue squares are absent teeth. Yellow squares are partially erupted teeth. Unfilled squares are present teeth. Red outlines indicate teeth absent which would not be recorded if wax bites were taken on a weekly interval. Arrows indicate some locations where teeth were absent for more than one week. (**E,F**) Histograms of time after exfoliation before eruption of the following tooth in days showing the mode to be 6 days.

3.4 Right to Left Symmetry characteristic for each animal

Since our ultimate goal was to deliver treatments to the geckos in order to study their effect on tooth replacement, it was necessary to first characterize in detail the normal patterns. Wax bite data each of the three geckos was analyzed for the following parameters: Right-left symmetry, the presence of waves of exfoliation, number of absent teeth per week and timing between exfoliation and replacement. These analyses were carried out for 7 weeks on the 3, unperturbed geckos.

We wondered whether it was possible to maintain individual tooth symmetry on the right and left sides in an animal with so many teeth. The null hypothesis is that there is no relationship between the right and left sides so each tooth exfoliates on its own schedule. The null hypothesis was rejected because individual tooth symmetry between the right and left sides was high when all teeth, both present and absent, were included in the analysis. The percentage symmetry was on average 91.84% in LG59 (SD ±4.78%), 87.35% in LG60 (SD ±5.68%) and 82.04% in LG 100 (SD ±7.69%) (Figure 3.3A, 1-7 week bars). When just the missing teeth were scored the symmetry between the right and left sides was lower at an average of 59.33% in LG 59 (SD ±21.82%), 55.28% in LG 60 (SD ±19.90%) and 37.77% in LG 100 (SD ±15.38%) (Figure 3.3B, 1-7 week bars). The exact tooth positions that are being shed are more variable but are more likely to be the same than not (except for LG100). This data suggests that there are biological factors regulating the timing of tooth shedding and that these are operating in a similar fashion on both sides of the jaw.

Figure 3.3 Right to left symmetry in the leopard gecko

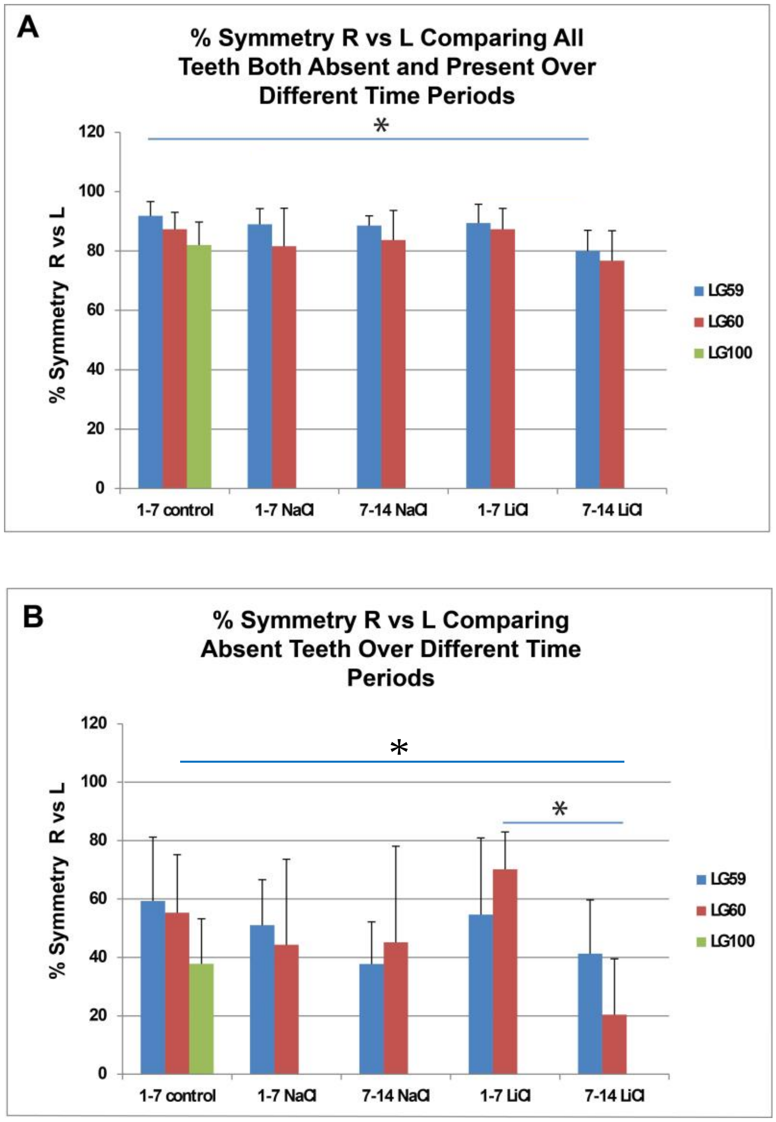

Fig. 3.3 – Right to left symmetry is illustrated in these bar graphs. (**A**) Bar graph comparing percentage symmetry of all teeth both absent and present in the control, NaCl treatment period, and LiCl treatment period in all three animals. Only LG59 had a significant break/decrease in symmetry between the control period, and 7-14 weeks post LiCl (asterisk = $p < 0.05$). (**B**) Bar graph comparing percentage symmetry of absent teeth in the control, NaCl treatment period and LiCl treatment period in all three animals. Only LG60 had a significant break in symmetry between the control period, the LiCl1-7 and 7-14 (asterisk = $p < 0.05$). Key: error bars = 1 standard deviation.

3.5 Posterior-anterior and right-left patterns of tooth exfoliation are visible in normal leopard geckos

The symmetry data suggested that there are underlying biological factors that regulate the exfoliation of teeth. To further explore this idea we investigated the pattern of exfoliation. The null-hypothesis is that teeth are exfoliated randomly at different intervals along the dentition. However, striking regularity was encountered. The absent teeth form a diagonal pattern when plotted over time and position in the jaw (Figure 3.4A-F). Exfoliation patterns in all three animals were observed to go from posterior to anterior, with every other tooth exfoliating each week and occasionally every two weeks.

We had the option of either scoring partially erupted teeth as being present or absent. When scored as present, there was no change in the waves of exfoliation. However, when we scored the partially erupted teeth as absent, several gaps in the posterior to anterior exfoliation pattern were filled in. In other words, patterns which would have been broken became complete. This was especially common in LG59 (Figure 3.4A,B) but less common in LG 60 and 100. Therefore for the remainder of the analysis we scored partially erupted teeth as absent.

Figure 3.4 Tooth replacement/exfoliation patterns during the control period

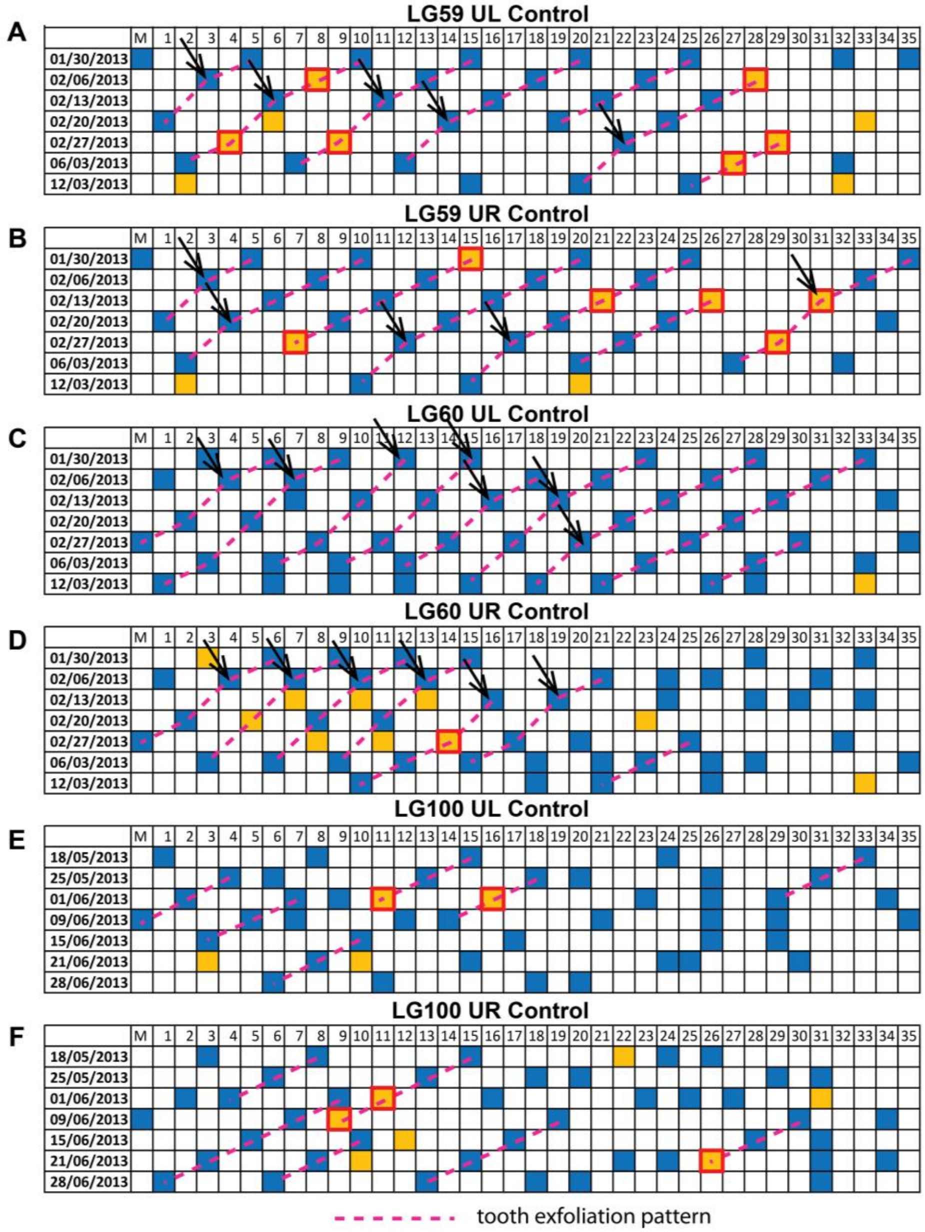

Fig. 3.4 – Records of wax bites taken during the 7 week control period on all three animals. (**A-F**) Illustration shows teeth absent and present over a 7week period on LG59, LG60 and LG100 upper left (UL) and upper right (UR). Rows indicate impression dates. Columns indicate tooth positions. Blue squares are absent teeth. Yellow squares are partially erupted teeth. Unfilled squares are present teeth. Red outlines indicate partially erupted teeth counted as absent which complete the exfoliation pattern. Arrows indicate locations where the exfoliation pattern switches from the pattern of exfoliation occurring weekly to every 2 weeks.

3.6 Timing of tooth replacement is characteristic for each animal

Phenotypic evaluation normally requires at least 3 biological replicates and usually these replicates are individual animals. If there was a high degree of variability between animals in the various parameters being measured, this would mean that we may not be able to detect phenotypes. The outcome of tooth replacement analysis would determine whether we would do a longitudinal or cross-sectional study.

The replacement time for each tooth across the jaw was relatively constant with on average one tooth replacement every 6 to 7 weeks. However, the number of teeth absent per week per quadrant varied between animals. Average number of tooth absences per week for LG 59 was 5.43 on the left (SD ±0.98) and 5.43 on the right (SD ±1.13), for LG 60 values were 7.43 on the left (SD ±1.81) and 7.57 on the right (SD ±2.23), for LG100 average absent teeth per week was 6.43 on the left (SD ±1.81) and 6.71 on the right (SD ±1.60) (Figure 3.5A-F). We next wanted to determine whether each quadrant could be used as a technical replicate and averaged to obtain the mean values or whether the data for the entire jaw should be used as one data set. The right and left side of the arch of the control period were compared to each other using the Mann Whitney U test. No significant differences in the total number of absent teeth each week were noted between the right and left sides of the arch within the same animal (p= 1.0 for LG 59 and 60, p = 0.79 for LG 100). There was no increase in accuracy obtained from averaging the technical replicates therefore we included the entire arch as one data set. A second result from the number of absences per week was that each animal maintained their condition over the 7 week control period but there were differences between animals.

Figure 3.5 Total number of absent teeth per week during the control period

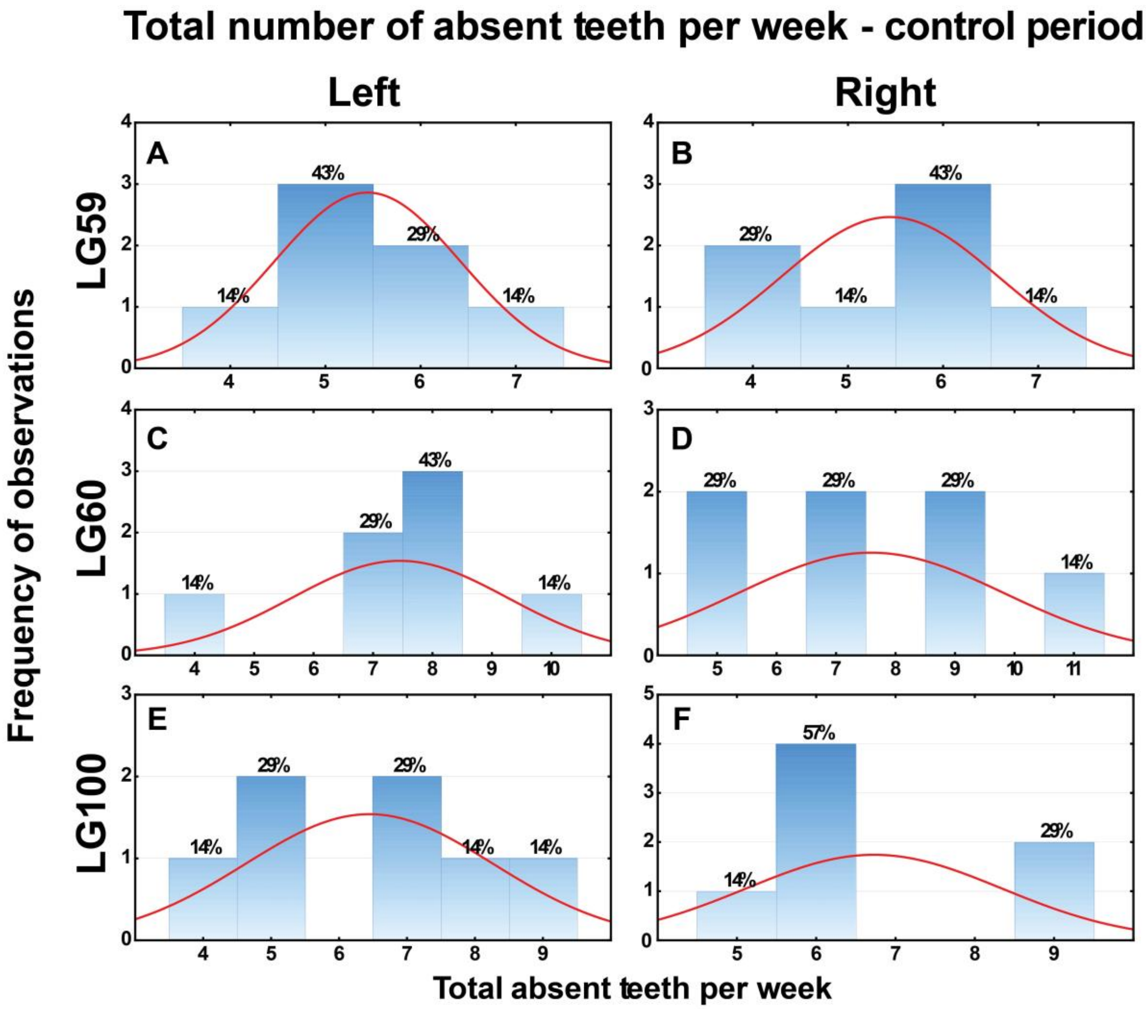

Fig. 3.5 – Comparison of total number of absent teeth per week of the left (**A,C,E**) and right (**B,D,F**) quadrants in the control, uninjected period. Histograms with the X axis indicating the number of teeth absent and the Y axis showing the number of weeks. The mode for the right and left sides were not equal in all three animals. LG59 never had more than 7 teeth missing per quadrant and the mode was between 5-6 teeth. LG60 had up to 11 teeth missing in certain weeks. LG100 had typically between 5-6 teeth missing per quadrant.

The time elapsed between exfoliation and eruption of the replacement tooth was on average 1 week with the rare tooth taking 2-4 weeks to replace (Figure 3.6 A-F). Thus teeth were obviously fully formed and already resorbing the tooth that was in the oral cavity, resulting in relatively quick turnover of the dentition. The right and left sides were also compared in terms of the length of time until eruption following exfoliation using the Mann Whitney U test. For all three animals, there were no significant differences in the timing of the exfoliation to eruption of a replacement tooth between the right and left sides of the arch ($p = 0.63$ for LG 59, $p = 0.35$ for LG60, $p = 0.56$ for LG 100).

Figure 3.6 Time before eruption of replacement tooth after exfoliation in the control period

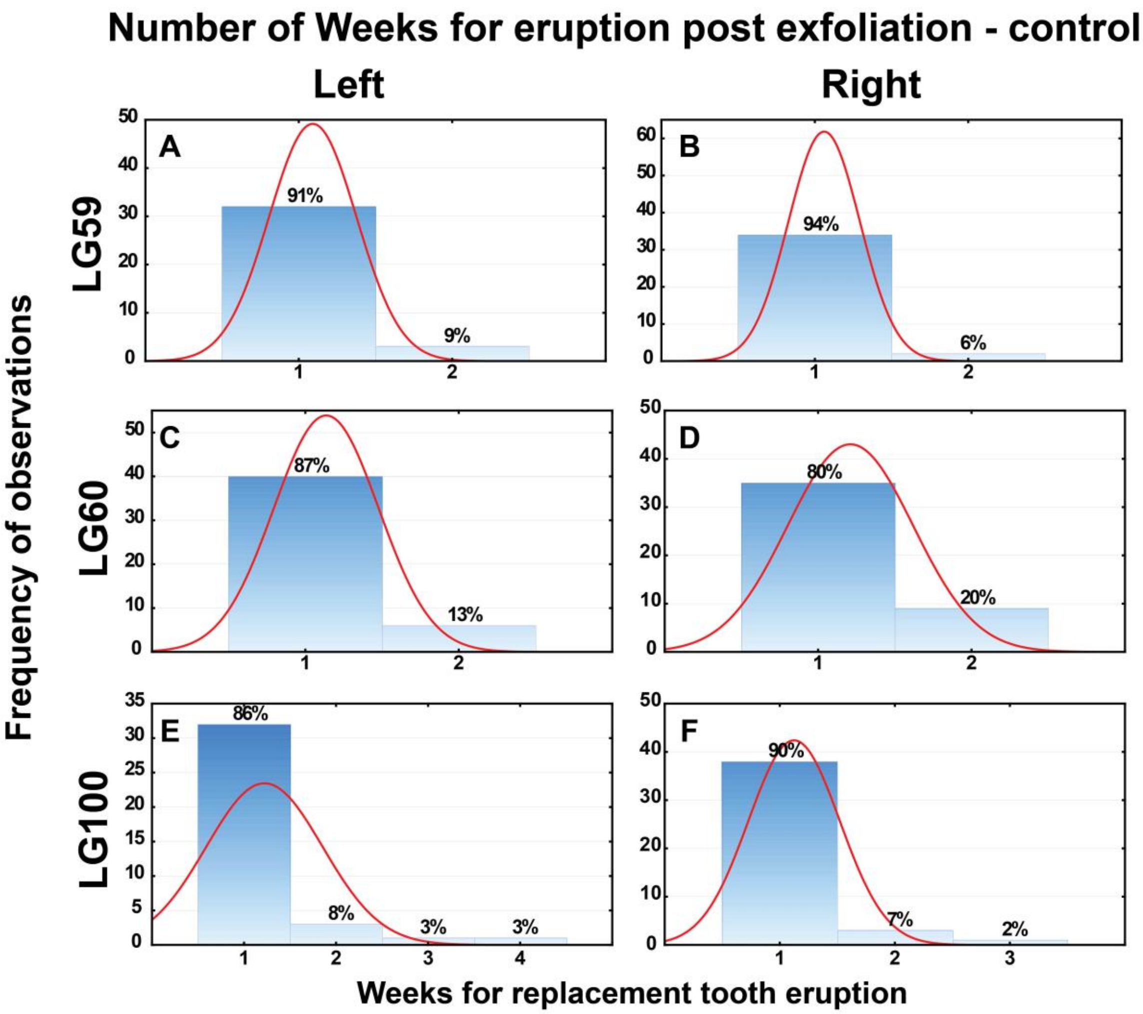

Fig. 3.6 – Comparison number of weeks for replacement tooth eruption of the left (**A,C,E**) and right (**B,D,F**) quadrants in the control, uninjected period. For all three animals the mode was 1 week for replacement teeth to erupt. This again validates our decision to take bites weekly, rather than every 2 weeks.

3.7 NaCl injections into the palatal shelves do not affect right to left symmetry, tooth absences or replacement time of lost teeth, but do affect tooth exfoliation patterns in the mid-posterior parts of the arch

Originally, we hoped to use each animal as a biological replicate, but as indicated above there was biological variation in between the animals. To compound this issue, one of the three geckos, LG 100, became sick and unfortunately had to be dropped from the study. Therefore, instead of using each animal as a biological replicate, we used each animal as its own control in a longitudinal study design. Thus we compared the effects of treatments all within the same animal as opposed to between animals.

The first experiment consisted of injection with NaCl. Here we tested whether the injection technique itself disrupted development. We examined whether NaCl would affect symmetry, tooth exfoliation patterns, tooth absences or replacement time following loss of teeth. Overall in both LG59 and 60, exfoliation patterns were posterior to anterior, with every other tooth exfoliating in waves. There were, however, differences from the control period. In LG59, there was a loss of exfoliation waves in the most posterior portion of the arch (near the site of injection). Some teeth did not replace for approximately 7 weeks post injection (Figure 3.7, NaCl-1, tooth positions 26-35) but then appeared to recover in the second 7 weeks after injection (Figure 3.7, NaCl-2). In LG 60 in the NaCl-1 period there was a delay noted to the exfoliation pattern at tooth positions 18-22 where the next tooth in the replacement wave took 3 weeks before replacing. There was also a loss of the exfoliation pattern at tooth positions 22-26. These disruptions mostly recover back to weekly waves in NaCl-2. There is also an increase in the number of waves following an every 2-3 week pattern in the mid-posterior parts of the arch when compared to the uninjected control period (Figure 3.8). These findings suggest that the needle may have contacted the dental lamina in the area of injection possibly delaying and/or disrupting the replacement waves of the gecko dentition. Fortunately, after waiting the length of one tooth replacement cycle (approximately 6-7 weeks, Figure 3.2) the gecko mostly recovered back to the normal replacement patterns post injection.

Figure 3.7 An overlay of the right and left side bite records for LG59 for the entire experimental period.

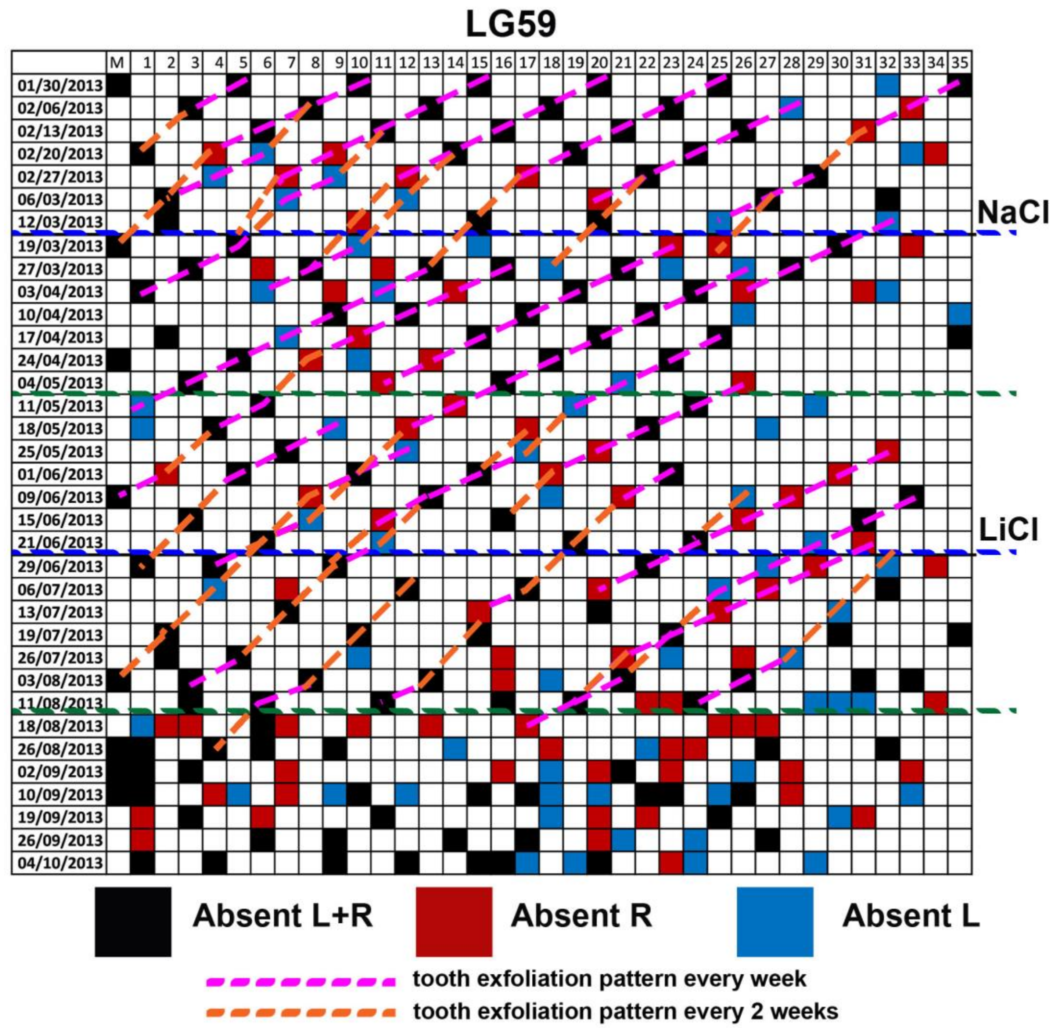

Fig. 3.7 – An overlay of the right and left side bite records for LG59 for the entire experimental period. Rows indicate impression dates. Columns indicate tooth positions. NaCl and LiCl injection weeks are indicated by the horizontal blue dashed lines. Forest green dashed lines show the separation of the NaCl and LiCl into sub groups of 7 weeks for analysis. Pink and orange dashed lines show the waves of tooth replacement. Note that all dashed lines are within a quadrant so that a blue and a red square are never connected. In the control period, the majority of patterns are alternating teeth being lost every week. In the first 7 weeks of the NaCl period, the same patterns continue but appear to have lost posterior waves. These recover in the second 7 weeks. Following LiCl, most of the waves throughout the arch are following the every 2 week pattern for the first 7 weeks. In the last 7 weeks, no waves could be drawn on the data.

Figure 3.8 An overlay of the right and left side bite records for LG60 for the entire experimental period

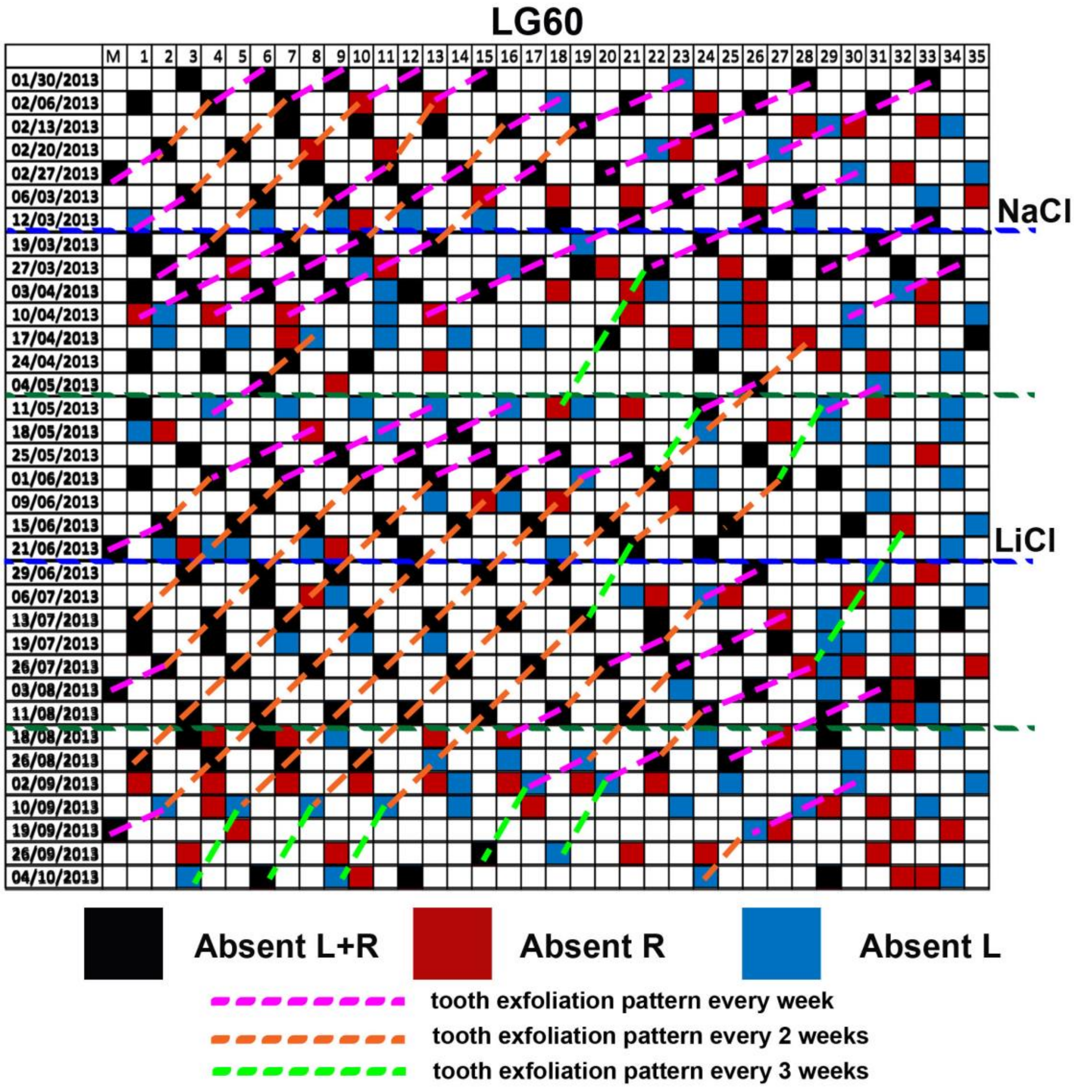

Fig. 3.8 – An overlay of the right and left side bite records for LG60 for the entire experimental period. Pink, orange and lime green dashed lines show the waves of tooth replacement. In the control period, the majority of patterns are alternating teeth being lost every week. In the NaCl period, the first 7 weeks appear to have lost waves in the mid to posterior with one wave mid arch following an every 3 week replacement/exfoliation pattern. These mostly recover back to weekly waves in the second 7 weeks but there is an increase in the number of waves following an every 2-3 week pattern in the mid to posterior. In the LiCl period the majority of waves are comprised of teeth being lost every two weeks rather than weekly. The length of the waves seems to be longer than in the control periods, extending for over 7 weeks. In addition waves with teeth being lost every 3 weeks appear throughout the arch rather than just the mid-posterior region.

Another test of whether the NaCl injection had disrupted development was to assess the right-left symmetry and to compare this to the uninjected control period. Using repeated measures ANOVA, there were no significant differences (p = 0.47 for LG 59 and p= 0.65 for LG 60). When only missing teeth were included in the symmetry analysis, the symmetry was again lower but no significant differences were noted from the control period (p= 0.18 for LG 59, p=0.75 for LG 60) (Figure 3.3 A,B). The NaCl therefore did not adversely affect the right to left symmetry.

The Kruskal-Wallis test was used to determine whether the tooth absence per week data from either NaCl period was different from the control. For both animals, there were no significant differences (Figure 3.9 A-F, p=0.12 for LG 59 and p = 0.90 for LG 60). For the NaCl time period, the time elapsed between exfoliation and eruption of the replacement tooth was again 1 week with the rare tooth taking 2-3 weeks to replace, identical to the control period. (Figure 3.10 A-F, p = 0.40 for LG 59, p = 0.28 for LG 60). Although the first analysis of the waves of exfoliation appeared to show some slight regional differences in patterning following NaCl injections, based on the lack of phenotypic change in the symmetry, tooth absences per week or timing for individual teeth to be replaced, there was no statistical reason to keep the datasets from the NaCl-1, NaCl-2 and the non-treated data separate. For this reason the subsequent comparisons to LiCl were made to the merged, uninjected plus NaCL-1, NaCl-2 data set.

Figure 3.9 Comparison of the control and NaCl tooth absences justifies combining the data into one control period.

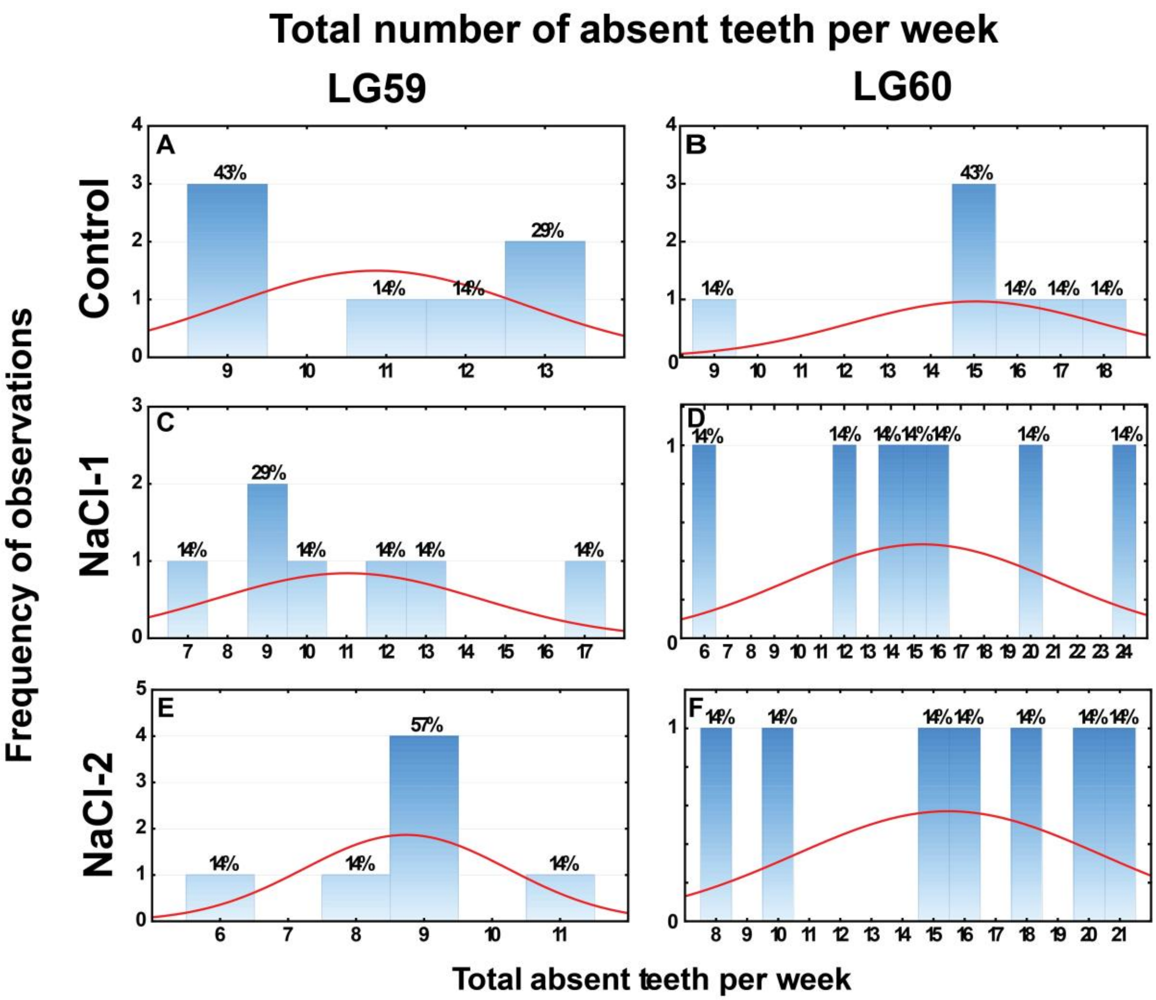

Fig. 3.9 – Combining the control and NaCl treatment periods is justified based on statistical analysis of tooth absences. Comparison of the total absent teeth per week for the maxillary arch between the control (**A,B**), NaCl-1(**C,D**), NaCl-2 (**E,F**) periods of LG59 and LG60. Comparing the mode in A, C, E shows that the most frequent observation was 9 teeth missing teeth for the arch in LG59 and this was not significantly different between the three periods. For LG60, the most frequent observation was 15 teeth absent for the arch in the control period. In the NaCl-1 and Nacl-2 periods the distribution of observations was even.

Figure 3.10 Number of weeks for eruption post exfoliation during the NaCl period

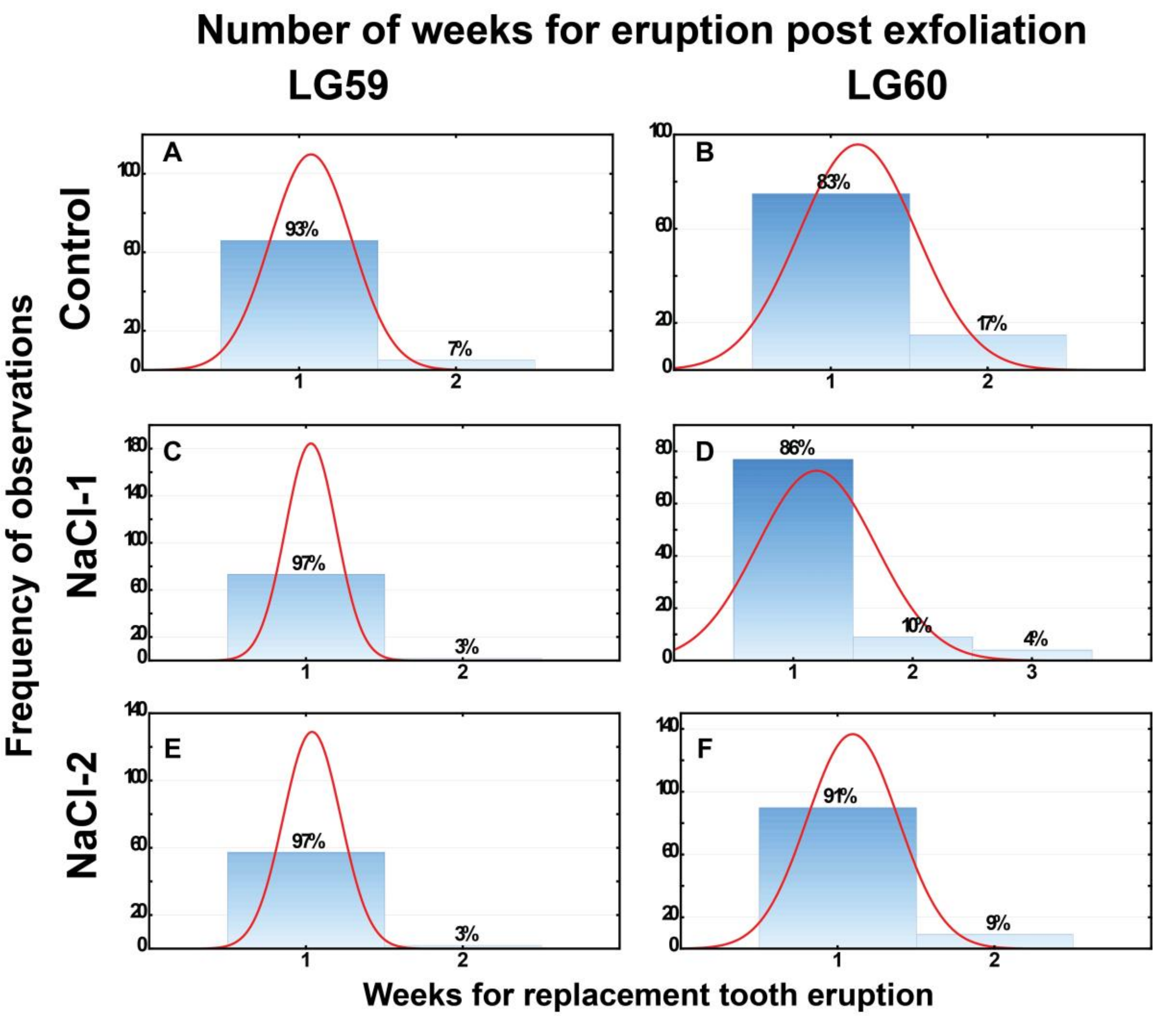

Fig. 3.10 – Comparison of number of weeks for replacement tooth between the control (**A,B**), NaCl-1(**C,D**), NaCl-2 (**E,F**) periods of LG59 andLG60. Both animals typically had 1 week elapse between tooth loss and emergence of the replacement tooth and this did not vary between experimental periods.

3.8 LiCl injections disrupt wave-like patterns of exfoliation and symmetry

Treatment with LiCl showed noticeable changes in patterns of exfoliation in LG 59 and less so in LG60. The 14 week LiCl period was also split into the first 7 weeks (LiCl-1) and second 7 weeks (LiCl-2) for comparison to the control period. In this way we could distinguish early from late effects of the LiCl injection. We first examined patterning effects of LiCl on the waves of exfoliation. These patterns cannot be easily analyzed using statistical approaches and therefore we are describing the changes in a qualitative sense. In LG59 and LG 60, the posterior to anterior exfoliation pattern of every other tooth was maintained through LiCl-1, but instead of the next tooth in the pattern exfoliating the following week; it took two weeks for the adjacent tooth to exfoliate (Figure 3.7, 3.8). In other words, the diagonal line drawn between exfoliated teeth over time formed steeper slopes. For LG60, in the mid (teeth 19-21) and posterior (teeth 28-32) regions of the arch, the next tooth in the pattern exfoliates every three weeks. Also, in the region of teeth 32-35, no pattern can be seen (Figure 3.8). This loss of pattern did not occur in LG59 where patterns appeared to be relatively normal in the mid-posterior region. It is possible this could be attributed to the actual injection into the dental lamina for LG 60.

In the LiCl-2 period, LG 59 showed a major change to the exfoliation pattern on both right and left sides (Figure 3.7) while LG 60 did not show such a change (Figure 3.8). The areas affected seemed to be more localized to the anterior/premaxillary region and also around teeth 17 to 27. There were larger groups of teeth absent at the same time (3 or more adjacent teeth) and teeth remained missing for longer periods of time causing a complete disruption to the exfoliation pattern. In LG 60, there are steeper waves due to it taking up to 3 weeks for the adjacent tooth to exfoliate and these waves carry on for longer periods of time (up to 7 weeks) (Figure 3.8). Overall, LG 59 showed a more exaggerated phenotype in response to LiCl including a complete loss of the exfoliation pattern throughout the arch 7 weeks after the LiCl injection.

A repeated measures ANOVA test was carried out to determine if there were any differences between the LiCl and the combined control/NaCl period. When all teeth, both present and absent, were included in the symmetry analysis, right to left symmetry for LG59 was affected in the LiCl-2 period but for LG 60 was close but overall not affected (Figure 3.3A). LG60's symmetry was close to being significant (p =0.05) and post hoc testing (Tukey's HSD test) was carried out. With post hoc testing, no significant difference was detected between control and the LiCl periods, although LiCl-2 was close (p=0.07). For LG 59, a significant difference was detected (p=0.015) and post hoc testing was also carried out. For LiCl-1, there was no significant difference from control (p=0.78), but for LiCl-2 there was a significant difference from the control (p=0.016), but not from LiCl-1 (p=0.054). LiCl therefore appeared to decrease the right to left symmetry of LG 59 during the LiCl-2 period. LG 60 did not have significant effects on symmetry when comparing both present and absent teeth.

Conversely, when just the missing teeth were scored, the limited right to left symmetry after LiCl injection for LG60 was affected but LG59 was not (Figure 3.3B). The repeated measures ANOVA test showed no significant differences between the combined control data and the LiCl injection period in LG59 (p=0.35). However, there was a significant difference in LG 60 (p=0.00004). Upon using Tukey's HSD test, the significant difference was between the control and LiCl-2 (p=0.001) and between LiCl-1 and LiCl-2 (p=0.0002). Right to left symmetry when just scoring missing teeth for LG60 is therefore also decreased during the LiCl-2 period.

3.9 Timing of tooth replacement is delayed following LiCl treatment

Next, we wanted to see whether LiCl had any effect on tooth absences per week. LiCl-1 and LiCl-2 were compared to the combined control/NaCl period (Figure 3.11 A-F), using the Kruskal-Wallis test. For LG60, there were no significant differences noted ($p=0.095$). For LG59, there was a significant difference in the number of teeth absent per week between the three periods ($p= 0.002$). Post hoc testing (Mann Whitney U tests) revealed the significant difference to be between LiCl-2 and the control period with $p = 0.0007$. Due to the increase in the number of compared groups by separating the LiCl period into two separate seven week periods, the Bonferroni Correction was applied. With this correction, the p value must be less than 0.017 (0.05 divided by 3 groups/hypothesis), for the result to be truly significant. The next question we asked was whether there were positional differences in the LiCl effects going from anterior to posterior.

Figure 3.11 Total number of absent teeth per week in the control/NaCl versus the LiCl period

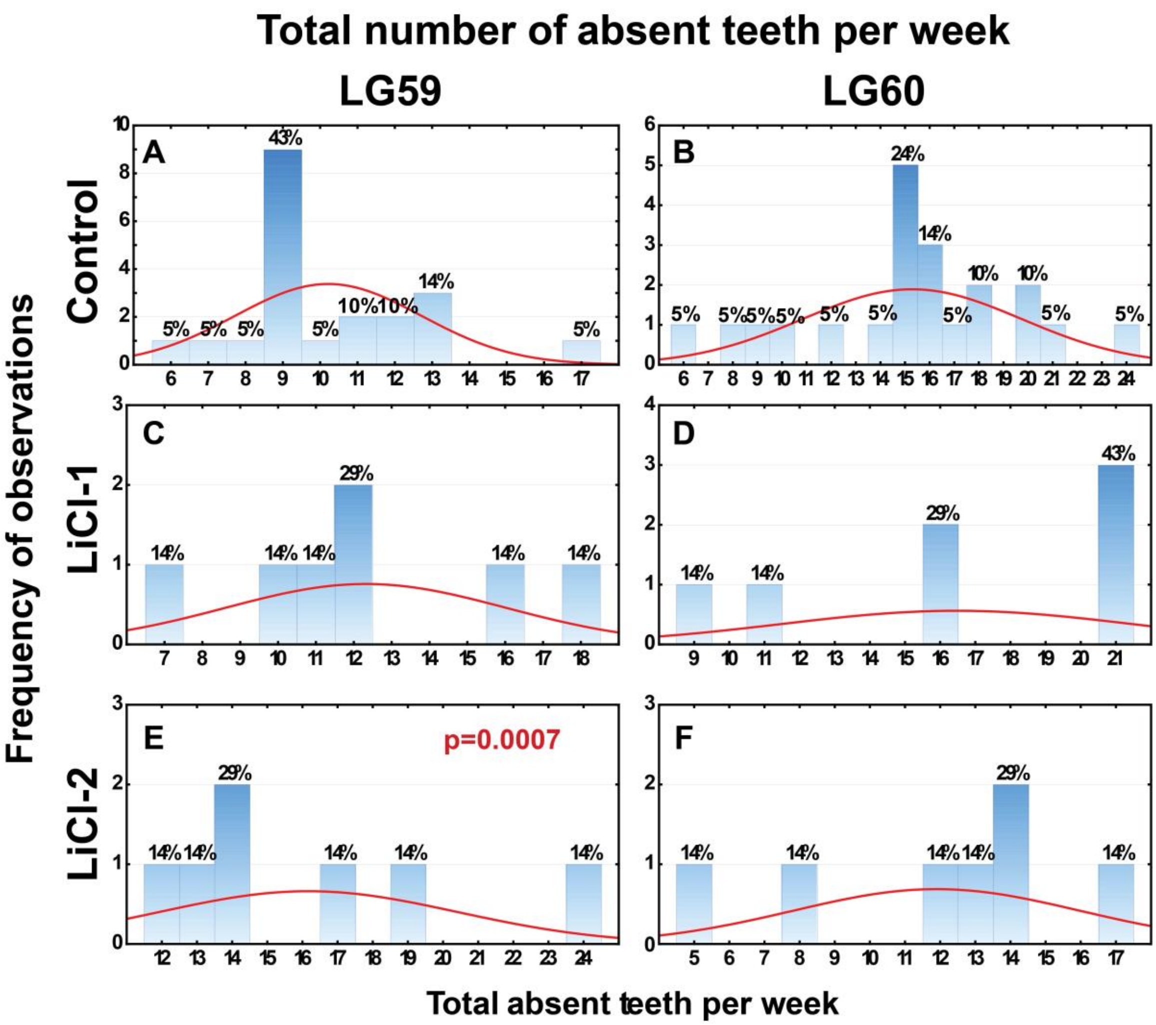

Fig. 3.11 – Comparison of total number of absent teeth per week between the control/NaCl (**A,B**) , LiCl-1(**C,D**) and LiCl-2(**E,F**) periods in LG59 and LG60. For LG59, there was a significant difference between the LiCl-2 and the control period (red color p-value). The mode for the control period is 9 absent teeth, whereas for LiCl-2 it is 14 absent teeth. LG60 has no significant difference between treatment and control periods.

3.10 Positional differences in the response to LiCl

We have already shown there are posterior-to-anterior waves of tooth replacement which suggests some type of jaw-wide pattering mechanism. Through dividing the arch into 5 segments of 7 teeth, we wanted to determine if any part of the mouth was more affected than another. For LG59, there was a significant difference noted (Kruskal-Wallis) in the anterior region, tooth positions 1-7 (p =0.006) as well as posteriorly, tooth positions 22 to 28 (p =0.02). Post-hoc testing (Mann Whitney U tests) showed a difference between the control and the LiCl-2 (p=0.0002 for teeth 1-7, p=0.005 for teeth 22-28). In addition, there was a significant difference in the anterior region (p=0.02) between LiCl-1 and LiCl-2. However, after applying the Bonferroni Correction (p=0.05 divided by 15, or 5 bins X 3 treatment periods = 0.0033), only the tooth positions 1-7 between the control period and the LiCl-2 was significantly different (Figure 3.12 A-E). The increase in the tooth absent number in the anterior region of the upper dentition could mean that either there was an increase in the number of individual replacements or more weeks elapsed after exfoliation before eruption of the replacement tooth. LG60 was analyzed in similar manner but there was no significant difference between periods by tooth position.

Figure 3.12 Total number of absent teeth per week for LG 59 during the LiCl period binned according to tooth position

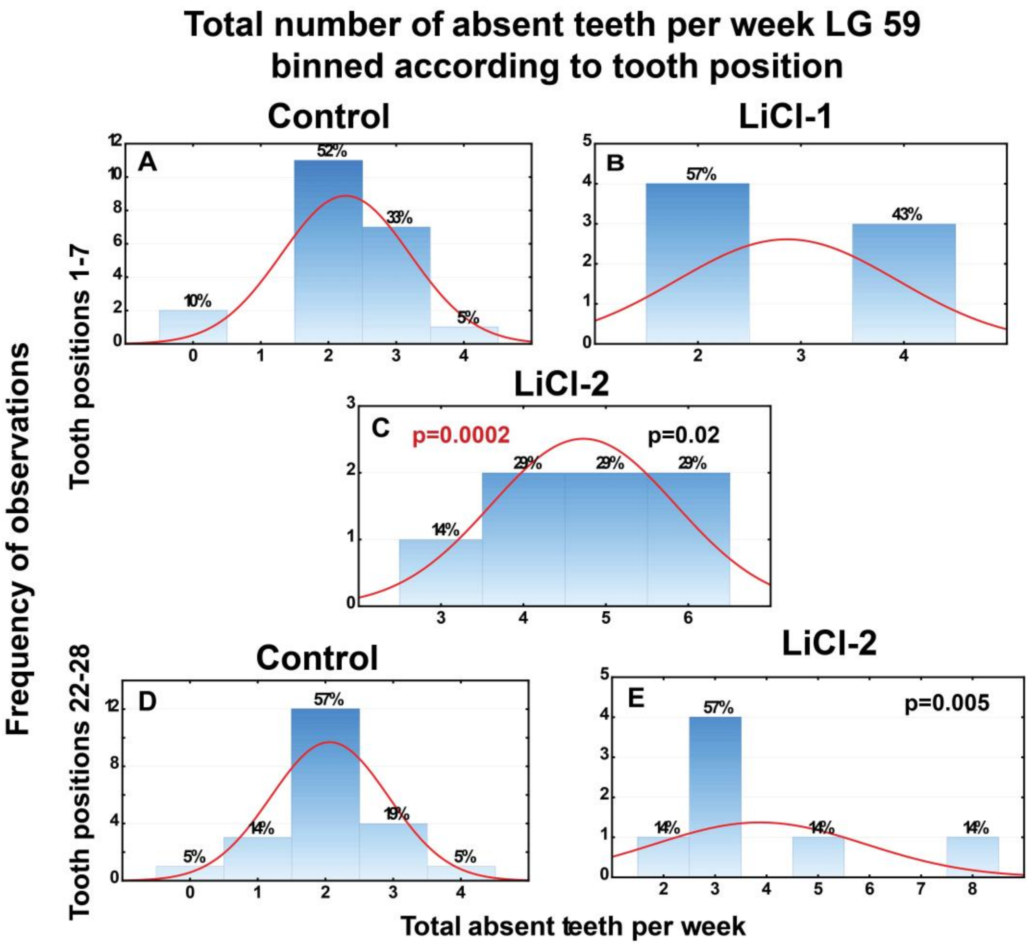

Fig. 3.12 – Comparison of total number of absent teeth per week for tooth positions 1-7 in the anterior and 22-28 in the posterior. A total of 5 bins were created for each quadrant each containing 7 teeth. In the anterior teeth, there is a significant increase in absent teeth between LiCl-2 (C) and the control (A) (red color p value). After Bonferroni correction, there is no significant difference between teeth absent in positions 1-7 LiCl-1 (B) and LiCl-2 (C); and teeth absent in positions 22-28 in control (D) and LiCl-2 (E). After correction where P.05/15 bins the p value must be less than $p = 0.003$ therefore LiCl-2 for tooth positions 22-28 and LiCl-1 for tooth positions 1-7 no longer reaches statistical significance (black color p value).

For LG 59, the time elapsed between exfoliation and eruption of the next tooth was significantly longer for LiCl-2 compared to control ($p=0.0073$, Bonferroni correction $p<0.017$). However the LiCl-1 period was close but did not quite reach significance compared to controls ($p = 0.02$) (Figure 3.13 A-F). When teeth 1-7 were tested for significance with Kruskal-Wallis and post-hoc Mann-Whitney U test, there was also significantly longer time after exfoliation prior to the eruption of the replacement tooth ($p=0.0003$, Bonferonni correction $p<0.0033$). LiCl-1 period did not reach significance compared to controls with Bonferroni correction ($p = 0.004$) (Figure 3.14 A-C). For LG60, there was no significant difference in the timing of the exfoliation to eruption of a replacement tooth between the LiCl periods and control periods ($p=0.05$). There are two possible explanations for the increased absent teeth in the anterior region of LG59. One is that there is an increase in time needed for replacement or the second is that exfoliation has sped up so there are more tooth shedding events captured.

Figure 3.13 Number of weeks for eruption post exfoliation in the LiCl period

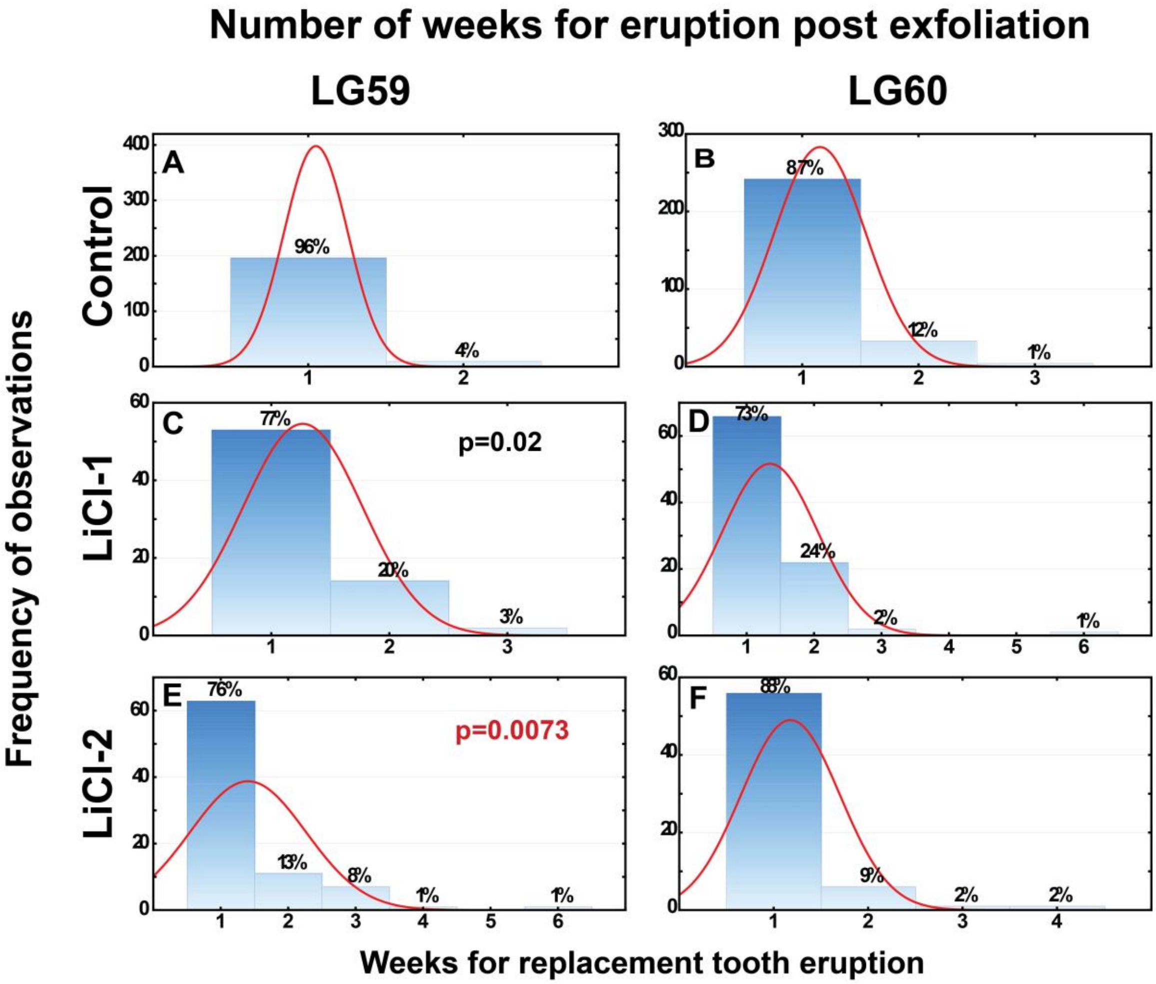

Fig. 3.13 – Comparison of total number of absent teeth per week between the control/NaCl (**A,B**), LiCl-1(**C,D**) and LiCl-2(**E,F**) periods in LG59 and LG60 Statistical significance after Bonferroni correction is shown by the p-value (red color). Values which are no longer significant after Bonferroni correction are shown by p-value (black color). LG59 has a longer period before teeth emerge in the LiCl-2 period.

Figure 3.14 Number of weeks for eruption post exfoliation in LG 59 during the LiCl period is prolonged in the anterior segment.

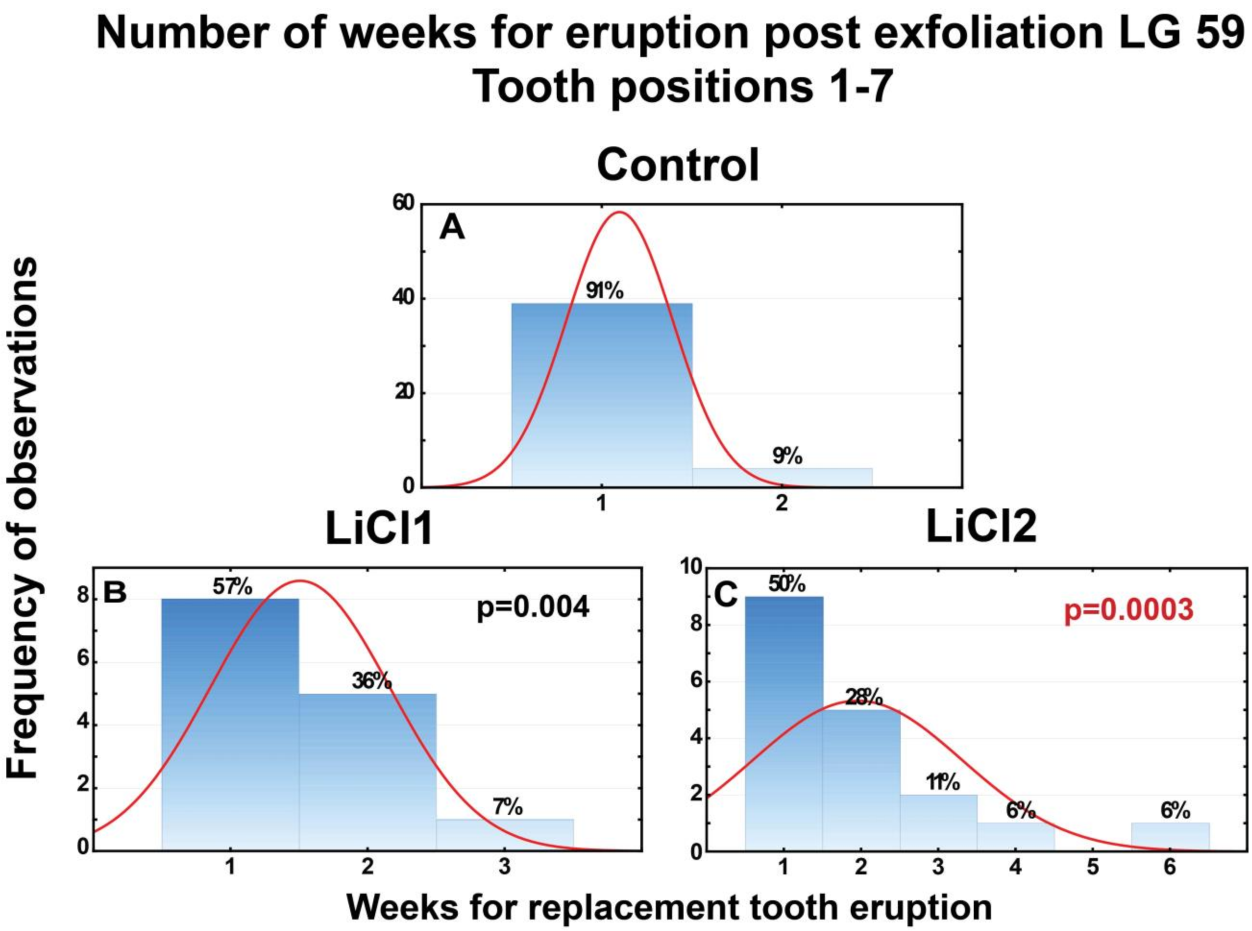

Fig. 3.14 – Comparison of total number of absent teeth per week in the anterior part of the maxilla. There is a significantly increased delay in eruption in the anterior segment in LiCl-2 period even after applying the Bonferroni correction (red color p value) but no significance in the LiCl-1 period (black color p value).

LiCl appears to have a significant effect in the anterior region of the mouth which causes disruption in the exfoliation pattern, number of tooth absences per week, tooth replacement timing, and symmetry as seen in LG59. Since LG60 appears to have a less prominent phenotype than LG59 after LiCl injection (symmetry of missing teeth, delay in exfoliation pattern), these results suggest possible variability between individual animals in their response to LiCl.

3.11 In vivo CT scan did not have sufficient resolution to see unerupted teeth

In vivo CT scans were done during the experimental period initially to determine whether the Zahnreihen could be seen while we were taking wax bite impressions to add another dimension to the phenotype analysis. However, even at the high resolution on the in vivo micro CT scanner of 50 µm, the scans were not clear enough to see partially formed, unerupted teeth or Zahnreihen. While this was unfortunate, we were able to compare wax bites taken one day before the CT scan to the teeth visible above bone in the CT scan (Figure 3.15 A-G). The scoring of the absent and present teeth was done separately and without comparing until both counts were complete (Figure 3.15B,E). Almost all the tooth positions matched perfectly (Figure 3.15A,C,D,F,G,H). There were discrepancies where teeth absent in the wax bite were erupted by the next day. We believe that the data are real and not due to technical errors in scoring either the wax bite or the CT scan. This idea is supported by the fact that in the week following the CT scan, the same teeth that were present on the scan were indeed present in the wax bite (data not shown). There was also one curious case in LG 59, where two teeth had erupted and shed in the 5 day period between the wax bites and this fleeting presence was captured in the CT scan (Figure 3.15B). Taken together, our wax bites are a sensitive, non-invasive way to record erupted teeth and no further information is gleaned from the in vivo CT scan. The extra radiation dose from the CT scan at the current resolution is not justified. Higher resolution scans pose unacceptable risk to health of the animal.

Figure 3.15 In-vivo µCT scan comparison to wax bite impressions

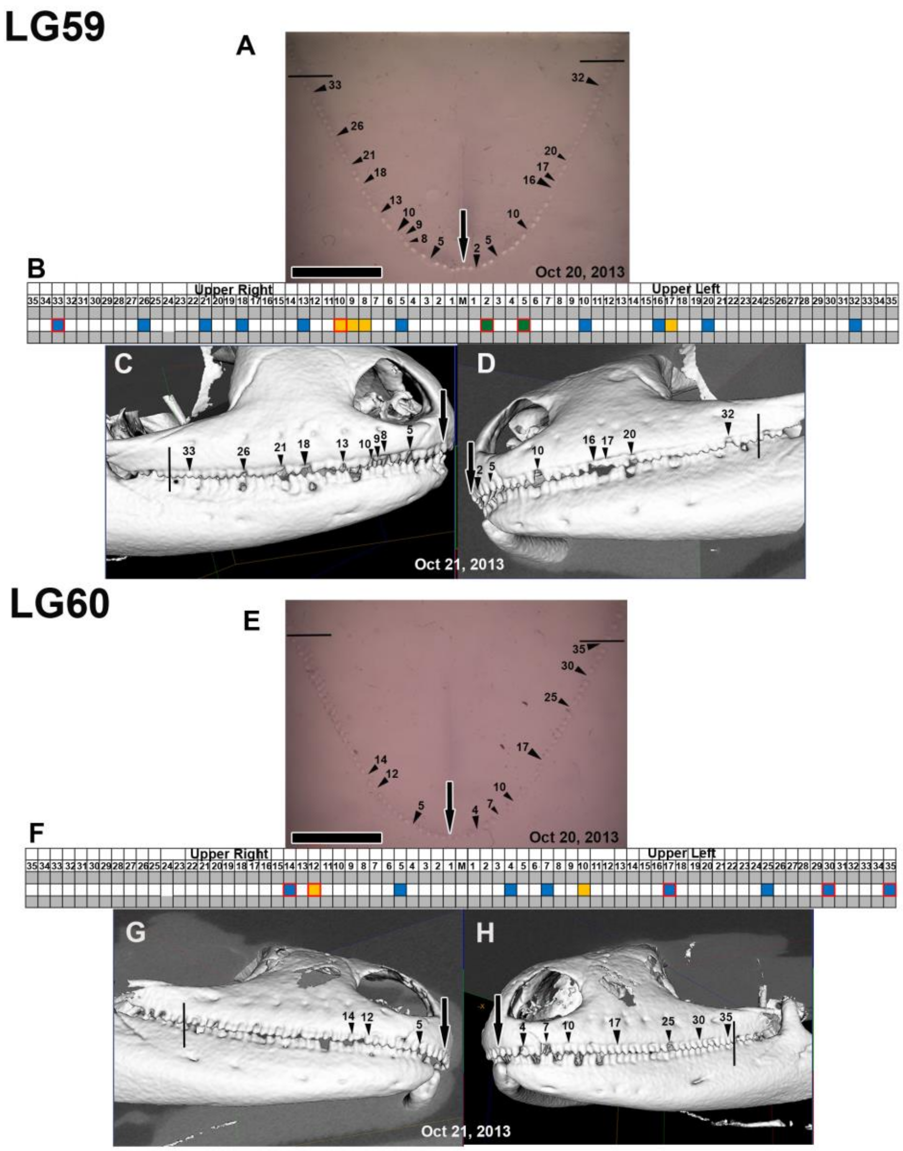

Fig. 3.15 – Comparison of the in-vivo CT scans with wax bite impressions taken one day prior to the scan for both LG59 and LG60. (**A,E**) Wax bites are shown with the midline marked (arrow), tooth 35 marked (black line on each side) and absent teeth marked. (**B,F**) Records of wax bites taken one day prior to µCT showing absent and present teeth. Columns indicate tooth positions. Blue squares are absent teeth. Yellow squares are partially erupted teeth. Unfilled squares are present teeth. Red outlines indicate teeth which are present on the CT scan but absent on the wax bite. Green squares indicate teeth which are present on the CT scan but absent in the wax bite taken 5 days after the CT scan (not shown). (**C,D,G,H**) µCT showing the midline marked (arrow), tooth 35 marked (black line on each side), and teeth which were missing on the wax bite for comparison. Scale bar = 5mm.

3.12 In vitro CT scans taken after LiCl injections show no changes in the Zahnreihen

A second set of LiCl injections was carried out on LG59 and LG60 at week 36 in October of 2013. However it became difficult to see the teeth in the wax bites for both animals. LG59 did not survive beyond a month after the second set of LiCl injections of wax bites and therefore the CT scan was carried out after death. Histology could not be carried out on LG59 due to the length of time elapsed between death and fixation. However, death did not interfere with in vitro CT scans. We investigated the scans to look for abnormalities in the teeth caused by the first or second set of injections. There were still many partially formed teeth and these were arranged in diagonal Zahnreihen (Figure 3.16A,A'). Therefore the first and second set of LiCl injections did not seem to affect general patterning of tooth development when viewed on a CT scan. There were no wax bites taken close to the time of death of the LG59.

LG60 was injected with LiCl in October as mentioned and then again in January 2014, one week prior to planned euthanasia. Thus the final CT scan was predicted to show more dramatic disruption to tooth patterning and perhaps a deficiency of partially formed teeth. There was no apparent deficiency or disruption in the unerupted teeth after 3 courses of LiCl treatment (Figure 3.16B). Therefore the drug was not overtly toxic to the cells. Interestingly, LG60 appeared to have some buccal bone loss on the upper right side and mandibular quadrants as seen on the in vitro CT scan post LiCl (Figure 3.16D and data not shown for mandible). It is possible that LiCl caused periodontal disease/bone loss or that the animal was suffering from metabolic bone disease which is a systemic disorder. We then examined the right and left sides of LG60 with histology.

The upper left side of LG60 displayed normal features of the gecko dentition. Coronal sections showed that teeth are more bulbous in the cervical region and coming to a point at the incisal surface (Figure 3.16C). In addition, there were 2 replacement teeth lingual to the erupted tooth (Figure 3.16C'). The dental and successional lamina can also be visualized. The buccal surface of the root is ankylosed to the bone. There is no bone on the lingual surface which is the condition in pleurodont dentitions. The erupted teeth of the gecko are approximately 250 μm in bucco-lingual width (Figure 3.16 C). The upper right side had bone loss in the CT scan which was also evident in histological analysis. The osteoid tissue where osteocytes were embedded, was less organized than on the left side (Figure 3.16 C",E,E',E"). This suggests that LiCl could also have an effect in the bone development and remodeling in the area or perhaps that the gecko had started to exhibit signs of metabolic bone disease around the time of euthanization. We were hand feeding the animal for the last 3 weeks of the experiment which could have impacted bone health.

Figure 3.16 In-vitro μCT scan and histologic analysis after LiCl injections

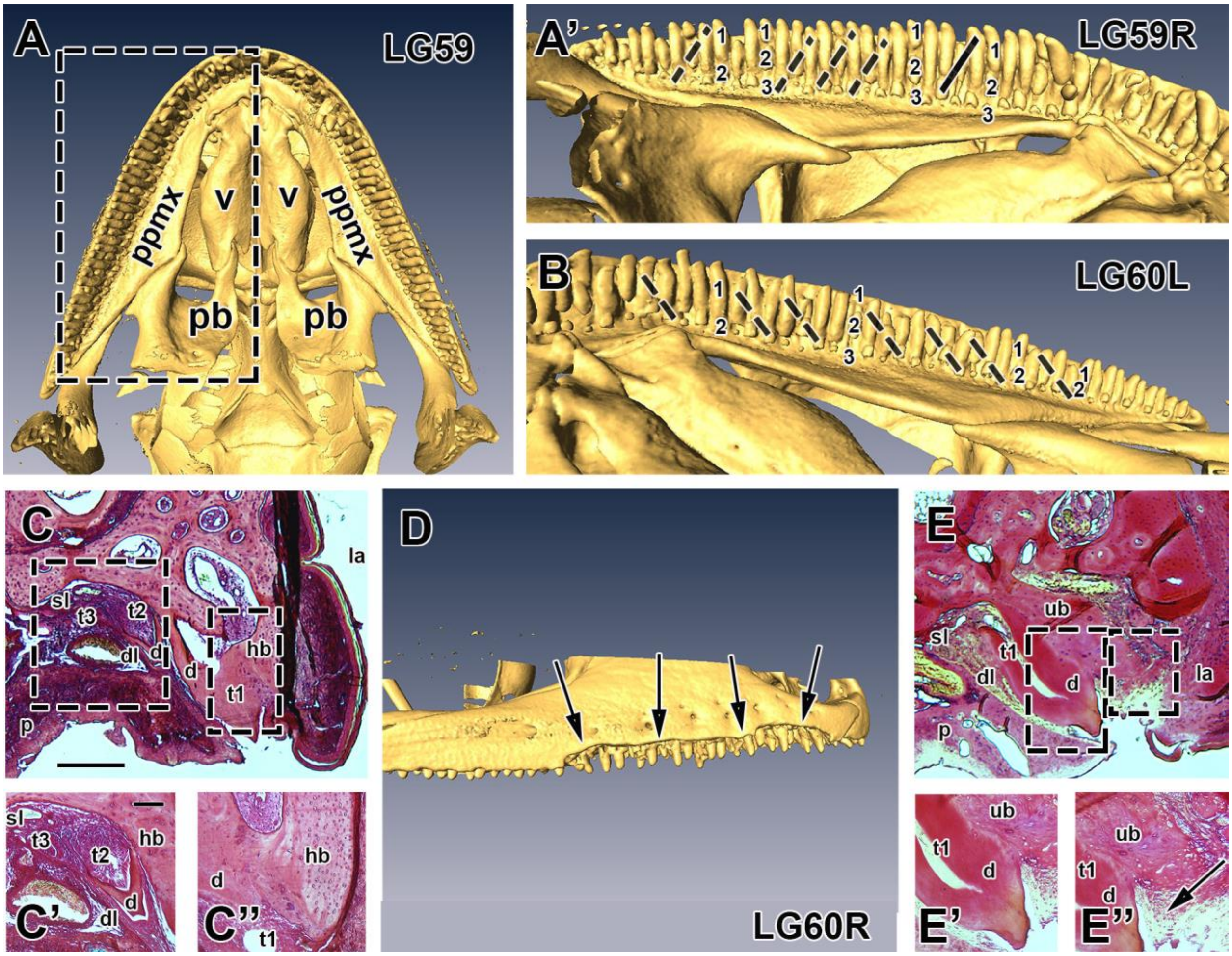

Fig. 3.16 – In-vitro CT scans and histologic analysis taken after LiCl injections. (**A**) CT isosurface showing the occlusal view of the maxillary arch of LG59, with continued development of generational teeth (numbered in **A'**) and Zahnreihen (diagonal dashed lines in **A'**). (**B**) Palatal view of LG60 upper left side showing continued development of generational teeth (numbered) and Zahnreihen also (diagonal dashed lines). (**C,C',C"**) Coronal sections through the upper left side of LG60 showing 3 generations of teeth. (**D**) CT reconstruction buccal view of the upper right maxillary arch showing significant buccal bone loss (arrows). (**E,E',E"**) Coronal sections through the upper right side of LG 60 showing abnormalities in the bone adjacent to the tooth attachment (**E''**, arrow). Key: d, dentin; dl, dental lamina; hb, healthy bone; la, labial surface; p, palatal surface; pb, palatine bones; ppmx, palatal processes of maxilla; pt, pterygoid bones; sl, successional lamina; t1, first generation tooth; t2, second generation tooth; t3, third generation tooth; ub, unhealthy bone; v, vomer; Scale bar for C,E = 250µm; Scale bar in C',C",E',E" = 100µm.

Chapter 4: Discussion

4.1 A high-throughput, sensitive and non-invasive means for longitudinal studies on tooth replacement in the leopard gecko

One of the main outcomes of my study was the development of a means to record the events of tooth shedding in real-time over many months. Although the number of animals involved was small, I was able to use them effectively to test out various methods and ultimately to arrive at one technique that could be used by others in future studies. Initially we tried using polyvinylsiloxane and red impression compound in custom trays. The reasoning behind this is that we might be able to have a more clear record with these dental materials. However, in order to take these impressions, the gecko had to be anesthetized with isoflurane since they would not accept the impressions while awake. This proved to be quite difficult since using these materials is very technique sensitive and having to anesthetize the gecko for each impression was inefficient.

After a suggestion from a visiting dental researcher, we decided to try wax and this was ultimately successful. Interestingly, we independently arrived at the use of baseplate wax without realizing that many decades ago, the same wax had been used to record teeth present in the slow worm (Cooper, 1966), green lizard (Cooper, 1964) and the green iguana (Kline and Cullum, 1984; Kline and Cullum, 1985). The specific details behind how to get the animal to open and close on the wax bite are not fully explained in those studies. I discovered that by touching the lower jaw the geckos would open wide long enough for the wax bite to be placed. These wax bites were so clear that we were even able to determine erupted, missing and partially erupted teeth. The inclusion of partially erupted teeth has not previously been done. The accuracy of the wax was further confirmed by matching the data with in-vivo micro CT scans. Recording partially erupted teeth ensures that absent teeth are not missed in between the weekly wax bite impressions.

We could have used radiographs to document tooth replacement as has been done in the alligator (Westergaard and Ferguson, 1987, 1990). However the overlapping nature of 2D radiographs makes it challenging to score the teeth. Multiple, offset views would need to be taken to see each tooth clearly. In addition, repeated exposure to x-rays is harmful and potentially hazardous to the animal. We have overcome the limitations of radiography and obtained dense data with the simpler method of wax bites.

4.2 Frequency of bite registration affects the data critically

Determining the time period at which to take wax bite impressions is essential to the accuracy of data set. If we did not take wax bites enough, we could miss out many absent teeth data points. If we took wax bites too often, we would have repeated data points. Our results of taking impressions once per week being the best for the least repeats and least omissions was in line with the previous studies on the slow worm (Cooper, 1966) and the green iguana (Kline and Cullum, 1984; Kline and Cullum, 1985). However, it should be noted that the periodicity of wax bites for iguana and slow worm was determine empirically. Indeed individual tooth replacement time differs greatly between the three animals (6-7 weeks for the gecko, 7-8 weeks for the slow worm, and 9-11 weeks for the adult green iguana). Not surprisingly the data for the iguana includes many repeated observances of the same tooth being absent (Figure 1.2). The data might be cleaner if the interval were increased to every 2 weeks, something that can be easily tested using the original Kline and Cullum dataset (Kline and Cullum, 1984). The leopard gecko appears to replace its teeth faster than other previously studied reptiles. The reasons for this are not fully known but possibilities include having smaller, more fragile teeth which may be more susceptible to wear and tear; having more teeth which would mean requiring faster replacement if the cycle progresses at the same speed as other reptiles; or being a younger age resulting in faster replacement (younger iguanas have teeth with a functional life of 6-7 weeks (Kline and Cullum, 1985).

The best example of where the frequency of bite registration may have affected our results is the negative effects of LiCl on anterior tooth replacement. In order to rule out whether LiCl had accelerated tooth replacement, recording the bite at shorter intervals would be required. In addition collecting cell proliferation frequency on LiCl versus control treated teeth would tell us whether LiCl had increased or delayed dental development. However, the majority of our data suggests that we had used the appropriate interval for wax bites for the entire study. First there was no difference in anterior and posterior tooth replacement rates during the control period. In addition, teeth were lost in the regular posterior to anterior alternating pattern indicating that LiCl had generally slowed down the entire replacement cycle. The only piece of data that suggests LiCl may have increased the rate of tooth replacement comes from the CT scans. In comparing the in-vivo CT scan with the two bites immediately preceding and following the scan two teeth in the anterior region were lost and replaced in the intervening 5 days. However this is only a limited set of observations for one brief period and for one animal. At present, we conclude that there is no support for the rate of tooth replacement in the anterior region becoming more rapid as a result of LiCl treatment.

4.3 Biological variability in the system

There are several sources of variability in our experiment. It is possible that the difference in phenotypes observed was due to variability in the injection technique. Perhaps in the case of LG59 the targeting was more successful than for LG60. A previous student in the lab had shown that the palatal injection technique brings the needle in close proximity to the dental lamina using dye tracking (Holmes, 2013). The dental lamina is accessible because the teeth are not covered by bone on the lingual. Therefore we are confident that the needle had reached the intended target at least for some of the injections (there were a total of 3 given). We also attempted to limit the diffusion of LiCl by first anesthetizing the palate with lidocaine and epinephrine. The epinephrine reduces blood flow to the area, allowing the local concentration to remain high for at least an hour, based on human responses to local anesthetic. The experimental design incorporated the best techniques available to us. Nevertheless each injection is unique and there is bound to be some redistribution of LiCl throughout the animal.

Each animal had slightly different patterns of tooth replacement even during the control period. Against this backdrop of variation in tooth shedding and emergence into the oral cavity, it is harder to determine whether there is an experimental effect. Ideally, we should have included more animals in the study; however there were many techniques that had to be optimized making it necessary to keep the sample size low in the first instance. My work has paved the way for more ambitious studies, now that we know how long to observe the animals and how to recognize a break from normal within the same animal.

4.4 LiCl delays tooth replacement leading to a change and/or disruption in the wave of exfoliation/replacement

The question that we can now answer is whether LiCl has an effect on tooth shedding in the gecko. It was clear that both animals had delayed tooth replacement although the phenotypes were different. In LG 59, tooth replacement patterns, tooth absence numbers and time before eruption of the replacement tooth after exfoliation were affected whereas in LG 60 only the tooth replacement patterns were affected. The effect of LiCl on the replacement pattern of reptiles has not previously been studied and through this research we have discovered that the effect is the opposite of what we initially hypothesized. Previous research from our lab indicate that LiCl activates the Wnt pathway using biochemical assays in the chicken (Geetha-Loganathan et al., 2014; Hosseini-Farahabadi et al., 2013) and gene expression studies on explants of snake teeth (Handrigan and Richman, 2010b). It was also shown in our lab that LiCl (Handrigan and Richman, 2010b) or another WNT activator BIO (Handrigan et al., 2010) induced proliferation in the reptilian dental epithelium. Activation of WNT signaling in dental epithelium of transgenic mice also radically increases proliferation and the number of teeth is hugely expanded (Järvinen et al., 2006; Wang et al., 2009). Our data contradicts the mouse studies. The reasons why LiCl has interfered with tooth emergence into the oral cavity are obscure since we were unable to assay the tissues for changes in cell proliferation. We also were not able to look at gene expression changes. It is certainly possible that complex feedback loops between signaling pathways are operating in the reptilian dentition (Handrigan and Richman, 2010b). Thus a gene that normally stimulates proliferation in the teeth may ultimately be repressed by the LiCl treatment giving rise to the phenotypes. Even without the underlying molecular mechanisms, the effect of LiCl is not immediate so we suspect the developing dentition is the target rather than the erupted teeth.

Our findings of LiCl delaying tooth eruption are in line with a recently published paper on zebrafish (Huysseune et al., 2014). These authors found that activating the Wnt pathway by adding LiCl to the water prevented the initiation of teeth in some fish and there was no effect on tooth replacement. The one consistent result was that there was no increase or acceleration of tooth replacement. However, no cell proliferation studies were done in their study either to determine possible cellular reasons for these results. Our results further support the idea that activating the Wnt pathway with LiCl does not accelerate tooth eruption. Based on the fish data, we presume that initiation of successional teeth is inhibited following LiCl injection in the gecko. We would need to carry out further histological and cell proliferation analyses to test this hypothesis.

There are several explanations for the delay in tooth replacement. One is that the animals were getting older and would have naturally slowed down the pace of tooth replacement. Previous studies on crocodilia by Edmund state that as the animal gets older, the replacement waves break down(Edmund, 1962). Studies tracking the replacement speed of teeth in the common lizard (Cooper, 1964), showed that tooth replacement in the young lizard was fast (2-3 weeks for replacement of an individual tooth) but as the lizard aged, the tooth took longer and longer to replace (up to 38 weeks by the lizard's adulthood). However, this explanation seems less likely for geckos since in lizards the replacement waves do not seem to break down throughout the lifetime of the animal which would happen as the rate of tooth replacement slowed (Edmund, 1969). Another possible reason for this delay in the exfoliation pattern could be seasonal differences in tooth shedding. Some reptiles do go through changes in speed of tooth replacement depending on the season (Cooper, 1966). The slow worm starts out with tooth replacement at a speed of one replacement every 6 weeks but by the autumn, the replacement speed has slowed down to once every 8 weeks. In order to evaluate this phenomenon in the leopard gecko, the exfoliation pattern requires to be followed for a longer period of time with no interventions. A third explanation is that the development of replacement teeth is slower, something that will need to be examined in the future with histological analysis. A fourth explanation is that LiCl affects bone biology, perhaps making the ankyloses more robust or by blocking osteo/dentinoclast function. Thus the erupted teeth would remain present in the mouth longer, leading to greater intervals between tooth shedding events. A fifth explanation is that the injection itself led to the changes in tooth replacement rate and pattern. There was a disruption and delay of pattern of exfoliation in the mid-posterior portion of the arch in both LG59 and LG60 following the NaCl injections but it occurred for a very short period prior to patterns mostly returning to normal. The injection itself could have contacted the dental lamina resulting in a mild disruption and delay in the exfoliation pattern prior to relatively fast recovery. This phenotype is localized in the mid-posterior portions of the arch and phenotypes found throughout the arch (as found with LiCl injections) cannot be attributed to the injection itself. Furthermore, there was no statistically significant disruption to the absent number of teeth per week, eruption of the replacement tooth and symmetry in the control or NaCl periods. The localized phenotype suggests that the technique of placing the needle is important if evaluating dental tissues in the mid-posterior parts of the arch since damaging the dental tissues may cause delay or disruption of the replacement pattern until recovery is possible. Lastly, physiological stress placed on the animal during injections could have caused a generalized disruption to the dental development and replacement pattern. If this were the case, we would expect to see identical disruptions in the waves of tooth replaced and other parameters following NaCl injections. However as the animals were largely unaffected by NaCl, it seems that stress was not a major factor in the study.

A final explanation for the LiCl effects observed could be that there are natural differences in the rate of tooth replacement in anterior versus posterior parts of the dentition. Cooper and Edmund (Cooper, 1964; Edmund, 1969) explore the possibility that the speed of replacement waves are not constant throughout the arch. They found that tooth replacement accelerates more towards the anterior making it possible to have tooth replacement taking place in the anterior but not in the posterior of lizards. If the anterior dentition in the gecko did replace at a faster speed, they could be more sensitive to the LiCl showing a more significant effect since more teeth would be developing at one time. There was only one animal that showed a specific effect of LiCl on anterior teeth so it would be necessary to repeat this experiment in more animals to be sure that this was a real effect. Nevertheless, as discussed before the bulk of the data does not support the idea that anterior teeth in the gecko have a faster turn over. .

4.5 LiCl affects the right to left symmetry of tooth replacement

The assessment of phenotypes in the post-LiCl treatment period using a breakdown according to time and position is original. We also created a new readout based on the initially high degree of R-L symmetry present in the jaws. Previous studies on many reptilian species had implied that R-L symmetry is not a common feature (Edmund, 1969). A recent study counted the total number of teeth on the right and left sides in crocodile, alligator and Komodo dragon (Brown et al., 2015). These authors found that the number of teeth in the premaxilla, maxilla and dentary were very similar on the right and left sides. In the alligator, the premaxilla had 5 teeth, maxilla had 15 teeth and dentary had 20 teeth most commonly. In the Komodo dragon, the premaxilla had 4 teeth, the maxilla had 13 teeth and the dentary had 13 teeth most commonly. We only had a sample of 3 geckos so it would be good to study this question in more depth with a much larger sample. The Brown et al. study used 61 alligator skulls, 23 crocodile skulls and 22 Komodo dragon skulls for their analysis. Our unique ability to record specific tooth positions over time will provide an added dimension to the analysis that was not captured in the Brown et al. study. Indeed a new collaboration between my supervisor and the Royal Ontario Museum has given us access to 20 additional gecko skulls that will be analyzed in the future.

Symmetry was broken clearly in LG 59 when all teeth were included, while symmetry was broken in LG60 when only missing teeth were included. When all teeth were included, LiCl had a significant effect on the R-L symmetry in the LiCl-2 period in LG59. When only absent teeth were taken into account, LiCl significantly reduced symmetry in the LiCl-2 periods in LG60. These findings supports the idea that LiCl affects the developing dentition since the phenotypes took 7 weeks to manifest which is the approximate lifetime of an erupted tooth. We acknowledge that our experimental design may have obscured even greater effects on symmetry. Both sides were injected with LiCl so there is likely to be more total variation than would normally be present. The rationale for injecting both sides was that we knew the LiCl would be redistributed systemically, even from a unilateral injection. However reflecting on the data, it would be interesting to refine the experiment by injecting one side with LiCl and the other with no intervention or control solution.

4.6 Lack of change in the Zahnreihen suggests the effects of LiCl were subclinical

Zahnreihen are present in the leopard gecko similar to previous findings in the iguana, lizards and alligator (Edmund, 1960; Edmund, 1969; Kline and Cullum, 1984; Kline and Cullum, 1985; Westergaard and Ferguson, 1987, 1990) . We were able to recognize them quite clearly in the in-vitro CT scans (Figure 3.1). We attempted to perturb the patterning with several sets of LiCl injections, most notably; LG60 had 3 sets of injections. However even after such intense exposure to LiCl, there was no notable change in the Zahnreihen; at least as seen with µCT (Figure 3.16), suggesting that while the LiCl did affect the replacement/exfoliation patterns, the overall effect on the organization/arrangement of the developing teeth was not affected at a macroscopic level. Several explanations are possible for the lack of obvious disruption in the unerupted, partially mineralized teeth. A technical issue could be the concentration of LiCl was too low to have an effect. We had used the maximum concentration and volume of LiCl that could be safely injected, however as mentioned the solution diffuses out of the area rapidly. A slow-release system would have been preferable however a device such as a mini-pump is not feasible in the mouth. Perhaps encapsulated drug using lipid nanoparticles could work. Other WNT agonists that are in the form of small molecules such as BIO could have been delivered to the animals. These lipophilic compounds could be applied using microscopic beads as has been done in the chicken embryo (Bhullar et al., 2015) and in organ culture (Buchtová et al., 2008; Handrigan and Richman, 2010a, b). Further development of the method will involve testing of some of these compounds in vivo.

Interestingly, LG60 had severe bone loss in two quadrants several months after LiCl injections. LiCl has previously been shown to have an effect on bone (Meng et al., 2015) causing inhibition of intramembranous bone differentiation. The lack of osteocytes within the bone in our study is probably due to the harsh acid treatment used for decalcification so we cannot evaluate that status of osteogenesis. It seems unlikely that such localized bone loss could be explained by metabolic bone disease which should have affected more bony structures and all parts of the maxilla and mandible. In terms of the effects of this bone loss on tooth replacement, the animal with bone loss (LG60) had the least disruption in tooth shedding pattern meaning the bone loss did not affect tooth replacement or perhaps that LiCl maintained the tooth replacement pattern. In addition, these in vitro CT scans were done a couple months after the wax bite part of the experiment was complete and therefore the bone loss should not have affected the wax bite results.

4.7 Cell proliferation and other molecular studies were unable to be completed

We were initially hoping to do proliferation studies with the histologic sections of the gecko's jaws similar to that which has already been done on the gecko in our lab (Handrigan et al., 2010) and in alligators (Wu et al., 2013). Indeed we had carried out a pulse-chase labeling of the animals to visualize both slow and rapidly dividing cells in the developing dentition. However we ran into difficulties when trying to decalcify the jaws. It took several rounds of treatment before sectioning could be carried out. Unfortunately, the nuclei were destroyed, precluding proliferation analysis. The EDTA decalcification method will need to be used in the future, although this is much slower. Future proliferation studies would be beneficial to elicit whether there is still proliferation in the dental lamina or whether LiCl has reduced the number of dividing cells, especially in the successional lamina.

4.8 The future of the leopard gecko studies

More studies recording the replacement pattern of the gecko and effect of LiCl are warranted to confirm the results from my study. More leopard geckos evaluated over a longer period of time should be included in future studies. In addition, evaluating the geckos across different seasons might be beneficial to determine if there are any differences between tooth replacements at different times of the year. Determining the optimal dosage for LiCl or using other more lipophilic compounds would also be helpful. Looking at *Lef1* and *Axin2* expression along with beta catenin translocation into the nucleus would be needed to confirm the activation of the Wnt pathway. Evaluating the wave patterning data with more complicated statistical methods may be beneficial for future. Of course a larger sample size is needed to confirm these results. We can estimate the power needed to detect differences from our current symmetry data.

In this study, we have described a novel animal model, the leopard gecko which can be used to model many aspects of human dental development. We have also described a non-invasive, relatively simple method to record tooth replacement. The analyses invented for this study will help to determine whether tooth shedding and emergence is affected over time and position in the jaw. The gecko may help us to better understand tooth replacement mechanisms and molecular controls in human normal tooth replacement as well as the tooth-bone interface in development and disease. Through determining which molecules may have a significant effect on tooth replacement, the control of tooth regeneration in humans may be possible in the not too distant future.

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