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Coordination of bilateral tooth replacement in the juvenile gecko is continuous with *in ovo* patterning

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Abstract

We performed a test of how function impacts a genetically programmed process that continues into postnatal life. Using the dentition of the polyphyodont gecko as our model, tooth shedding was recorded longitudinally across the jaw. We compared two time periods: one in which teeth were patterned symmetrically *in ovo* and a later period when teeth were initiated post-hatching. By pairing shedding events on the right and left sides, we found the patterns of tooth loss are symmetrical and stable between periods, with only subtle deviations. Contralateral tooth positions shed within three to four days of each other in most animals (7/10). A minority of animals (3/10) had systematic tooth position shifts between right and left sides, likely due to changes in functional tooth number. Our results suggest that in addition to reproducible organogenesis of individual teeth, there is also a neotenic retention of jaw-wide dental patterning in reptiles. Finer analysis of regional asymmetries revealed changes to which contralateral position shed first, affecting up to one quarter of the jaw (10 tooth positions). Once established, these patterns were retained longitudinally. Taken together, the data support regional and global mechanisms of coordinating tooth cycling post-hatching.

Keywords

dentition; fluctuating asymmetry; reptile

INTRODUCTION

Teeth are cycling organs, shedding and replacing functioning teeth with newly formed ones long after the embryonic period in most vertebrates (Tucker and Fraser 2014). Amniote tooth replacement is highly patterned, most strikingly in extant and extinct reptiles with waves of tooth shedding passing through the jaws at regular intervals (Romer and Price 1940; Edmund 1960). Stem mammals such as the cynodont *Thrinaxodon* also show these patterns (Crompton 1963; Hopson 1964; Abdala et al. 2013); therefore, living mammals likely retain aspects of this patterning even though it is more difficult to detect in their significantly reduced tooth numbers. Although pre-molecular models for tooth replacement have remained prominent in the literature (Edmund 1960; Osborn 1970; Osborn 1998) and

much is known about individual tooth development (Balic and Thesleff 2015), the developmental basis for replacement patterning remains unknown. Nevertheless, patterns of tooth replacement represent an accessible, post-hatching record of the regulation of organogenesis. They provide a unique system to investigate the role of the environment in organ cycling, and the relative developmental independence of serial renewing units.

The defining trait of bilaterian animals is symmetry in right and left sides of the body, dictated by symmetrical gene expression about the mediolateral axis. Bilateral organs, especially those that develop post-embryonically such as secondary sex characteristics and cycling epidermal organs, may experience slightly different environments on each side that stochastically cause the same genetically controlled processes to manifest differently. Symmetry between such features may show three main patterns: embryonic, genetically-based symmetry may be carried forward and maintained (Swaddle and Witter 1997; Kellner and Alford 2003), it may degrade due to metabolic and environmental exposure (Hallgrímsson 1993), or post-embryonic bilateral features may acquire symmetry by a novel synchronizing mechanism (Swaddle and Witter 1997).

Teeth are ideal for connecting bilateral phenotypic variation to post-embryonic influences on development (Laffont et al. 2009). Because they do not continuously grow or remodel from the embryonic period like most traits, teeth are not confounded by scaling concerns, and past environmental perturbations may be erased with every new tooth germ (Tomkins 1999; Kellner and Alford 2003). In the embryonic dentition, teeth are initiated with a symmetrical spatiotemporal pattern (Osborn 1971; Westergaard and Ferguson 1990). Expression of many of the known tooth patterning genes, including *Shh*, *Sox2*, and *Bmp4*, is symmetrical in the mouse embryo (Sharpe 1995; Cobourne and Sharpe 2003; Juuri et al. 2013) and in other amniote embryos (Buchtová et al. 2008; Juuri et al. 2013; Wu et al. 2013; Lainoff et al. 2015). This includes expression marking rudimentary teeth in the diastema of the mouse (Peterkova et al. 2002; Klein et al. 2006) and along the marginal dentition of the alligator embryo (Harris et al. 2006; Weeks et al. 2013).

The formation of replacement teeth follows the basic steps and signaling pathways involved in embryonic tooth induction and morphogenesis (Stock 2001; Fraser et al. 2010; Balic and Thesleff 2015). The dental lamina, to which unerupted teeth are connected in most amniote embryos, is continuous around the jaw and provides a means for intra-arch regulation of cycling. Much less is known about post-natal dental patterning and symmetry. Bilateral symmetry of tooth emergence and root formation are apparent in individual humans (Grøn 1962; Suri et al. 2004) and highly heritable in twin studies (Hughes et al. 2007; Bockmann et al. 2010; Woodroffe et al. 2010). This genetic integration of post-natal phenotypes does not allow one to distinguish whether symmetry of tooth cycling dynamics is a remnant of embryonic patterning events or whether there is ongoing synchronization between right and left sides of the mouth of older individuals.

The large numbers of teeth in reptiles allow for the visualization of replacement patterns that are harder to detect in more limited mammalian dentitions, such as waveforms (Romer 1956; Edmund 1960; Edmund 1962; Cooper 1966; Osborn 1971; Kline and Cullum 1984; Kline and Cullum 1985; Kieser et al. 1993; Sassoon et al. 2015). In addition, the continuous dental

lamina persists into adulthood and connects tooth positions laterally. Using a polyphyodont model, the leopard gecko (*Eublepharis macularius*), we use bilateral variation to ask 1) if environmental factors dominate the dynamics of tooth cycling, 2) whether teeth are individually renewing units, or 3) whether there exists some mechanism that synchronizes tooth renewal within the jaw.

The leopard gecko is a tractable model for tooth renewal that can be investigated from oviposition through adulthood in the lab. The access to greater numbers of geckos than with many other reptiles permits the detection of individual variation in tooth replacement. The first three tooth generations are patterned during the last half of incubation, with all subsequent teeth initiated after hatching (Fig. 1). In this study, we use these two distinct time frames to observe both the patterning of the initial tooth row and post-hatching tooth replacement dynamics. We investigate whether embryonic teeth break symmetry and then test the hypothesis that in older animals, tooth positions are independently regulated developmental units, by examining bilateral variation in individual tooth positions over time. We look for evidence of covariance among tooth positions within a jaw quadrant and between antimeres, and for the influence of *in ovo* conditions on adult patterning.

MATERIALS AND METHODS

Study Animals

Leopard gecko (*Eublepharis macularius*) eggs and juveniles were obtained from private breeders in Surrey, British Columbia. Eggs were incubated in the lab at 28°C and 55–65% humidity. Rearing conditions for the juveniles (N = 10) included 12-hour light cycles, 21°C room temperature, and individual terraria containing 30°C heating pads and moist and dry hides. Growing animals were fed mealworms on a graduated schedule working up to three per day. Animals were obtained within the same breeding season. Work was approved by the UBC animal care committee under protocol A15-0242. Daily animal care was provided by the authors. Assessment of gecko health was made monthly by a UBC veterinarian. See SI Materials and Methods for further information on the model.

Micro CT scanning of hatchling leopard geckos

Within 24 hours of emerging from the egg, a separate group of hatchlings (N=5) was euthanized, fixed in 4% paraformaldehyde and scanned with μ CT (Scanco μ CT 100 Switzerland; 17.2 μ m isotropic voxel size, 55kVp energy, 200 μ A current, 850 ms integration time, and a 0.5mm aluminum filter). Several eggs were part of another study in which a small volume of dimethylsulfoxide (DMSO) was injected every other day for the two weeks leading up to hatching (N = 3), but these individuals displayed no detectable abnormalities. Mineralizing teeth from as early as cap stage (Handrigan and Richman 2010) were identified by visualizing isosurfaces, choosing the lowest threshold that still eliminated ambient noise in Amira (v. 5.4; FEI, Hillsboro, USA). See SI Materials and Methods for further phenotyping details.

Wax bite impressions and identifying tooth positions

Starting at one month post-hatching, wax bite impressions were recorded twice weekly up to eight months of age (baseplate wax #H00812, Coltène Whaledent, Inc, Cuyahoga Falls, OH, Table S1, Fig. 2A, Fig. S1A for study timeline). Maxillary impressions were photographed and analyzed because the tongue obstructed the mandibular impressions. Consensus dental arches recording the spacing of tooth positions along both sides of the jaw were used to bring homodont tooth impressions into proper registration and correctly identify contralateral tooth positions between observations for a given animal (Fig. 2E, Fig. S1B). This consensus arch with all possible tooth positions was created for each month and overlaid on individual wax impressions. Individual impressions were then scored for tooth presence or absence at each position, with partially erupted teeth scored as a separate category. There was no technical bias in the number of maxillary tooth absences recorded (e.g. uneven placement of wax, uneven occlusion; see Table 1 for recorded sample sizes, paired t-test nonsignificant between right and left sides, $df = 19$).

Arrays of presence/absence data were constructed by stacking tooth row data over time. We examined two 2-month periods corresponding to *in ovo*-patterned tooth initiation events (early period) and post-hatching tooth initiation events (late period). Arrays were truncated posteriorly at 30 tooth positions due to technical factors in capturing the posteriormost teeth. Injuries to teeth ($N = 16$ incidences) were identified within the arrays by laterally adjacent tooth positions with a partial or absent score, or by unusually long temporal runs of absences/partial impressions. All of these incidences occurred on the right side of the mouth due to the right-handed investigators' technique. We did not, however, find any other evidence of directional bias in tooth shedding asymmetry that could be attributed to investigator handedness as has been documented by others (Helm and Albrecht 2000). Broken teeth were rescored as present because they do not represent biological shedding events. All other partial impressions were scored as absences for subsequent quantitative analyses of tooth shedding events.

Quantification of shedding phenotypes

Each animal was studied longitudinally by examining dentitions that consisted of teeth that were patterned in the egg to dentitions after these pre-hatching teeth were shed and replaced. We quantified several parameters of the tooth replacement cycle: duration of tooth absence, shedding interval, and shedding event (Fig. 2B and Table S2). Percent asymmetry in tooth shedding events was calculated for each animal (percentage of total tooth positions in the premaxilla, anterior maxilla and posterior maxilla with asymmetry). A mixed-factor ANOVA was carried out to determine significant differences in percent symmetry by jaw region, observation period, and qualitative asymmetry motif. Shedding events were further characterized as bilateral events that could be symmetrical, isolated to a single side, or polarized with one side leading or lagging the other. The total number of polarized, isolated and non-classifiable events was determined per animal and converted to a percentage of total scored events. Bilateral shedding events were evaluated for independence under a binomial model of tooth shedding ($p = 0.5$ of seeing right or left shedding first, $n =$ number of polarized events identified per animal). See SI Materials and Methods for analytic details.

RESULTS

To evaluate the relative contribution of genes and environment on tooth cycling, we compare pre-hatching dentition phenotypes to post-hatching dentition phenotypes (Fig. 2A). Within the egg, environmental influences on the dentition are moderated, whereas post-hatching the dentition is functional and the animal is responding to more changeable circumstances. Changes to cycling or symmetry between the two periods would indicate a substantial environmental influence on the dentition as a whole.

Characteristics of the gecko dentition at hatching

At hatching there are 12–19 erupted teeth per maxillary quadrant (Fig. 1A; 16.1 ± 2.56 SD across 5 animals or 10 maxilla plus premaxilla quadrants). Several more tooth positions are patterned in the egg but are not a part of the functional tooth row at hatching. Rudimentary teeth are much smaller and located interstitially, superficial to the bone at the jaw margin (4–10 per quadrant; Fig. 1, inset). Over the first month of life, other developing teeth fill the spaces left by the egg teeth, as well as interstitially between erupted and rudimentary teeth.

Right-left symmetry is established prior to hatching, with contralateral tooth positions easily identifiable by their position and relative sizes (Fig. 1A). The right-left difference in number of marginal teeth was 2.3% ($\pm 1.7\%$ SD, $N = 21.7 \pm 1.04$ positions per maxillary quadrant, 10 quadrants). When including the mineralizing, unerupted teeth, symmetry differences set up prior to hatching were 0.8% ($\pm 0.8\%$ SD, $N = 70.6 \pm 1.82$ teeth per maxillary quadrant, 10 quadrants). The small variations in symmetry usually involved the most immature replacement teeth or rudimentary teeth. Order decreases in the posterior jaw, as tooth families become harder to distinguish. Neither total mineralized tooth number nor marginal tooth number in hatchlings correlates very strongly with hatchling maxilla length ($r = 0.55$ and $r = -0.33$, respectively).

Post-hatching tooth shedding faithfully records portions of the tooth replacement cycle

In the 10 hatched leopard geckos, teeth erupted more quickly during the early period (1–2 months after hatching; Fig. 2C) compared to the late period (6–7 months post-hatching; Fig. 2D). In the early period, 67% of the absences (tooth shedding events) were fully captured in a 3–4 day window whereas in the late period, absences extended into the next wax impression nearly 50% of the time (Fig. 3A). Rarely was an absence recorded over three observations (10–11 days, Fig. 3A). The average duration of a tooth absence increases from 4.74 days to 5.50 days ($p = 0.0158$, paired t test, $df = 9$).

The duration of a tooth absence appears to be slightly longer than the time between wax impressions, and we expect that we captured most shedding events in at least one wax impression. Notably, the record of tooth shedding creates a waveform pattern (Fig. 2C,D). Tooth shedding as recorded with wax impressions can therefore be considered a good proxy for a portion of the tooth developmental cycle (Fig. 2B) and we interpret dynamics to reflect true biological signals and not short-term behavioral or technical influences leading to premature tooth shedding.

Variability in the period of tooth turnover has no consistent pattern between periods

We began with an analysis of each animal and compared it back to its dentition established pre-hatching in order to test the effect of the environment on shedding characteristics. Shedding interval lengths are strongly modal for most individuals with side-pooled individual means varying about the corresponding time period averages (Fig. 3B). This modality in individual distributions is apparent at early and late periods, with most shedding intervals falling within a single number of wax bite observations (animals C, D; H early; J late) or split evenly between two consecutive numbers of observations. The proportions of outliers were also comparable (Fig. 3B). There are a few short shedding intervals in all animals, but for early periods where more intervals are captured, the next shedding event almost always falls within the waveform pattern (Fig. S3–S4). Either the anomalously short shedding interval is followed by another short shedding interval, or followed by a particularly long shedding interval.

We hypothesized that with increased age, animals would have accumulated more noise in the replacement system leading to greater variance in the shedding interval. However, there was no consistent pattern to the change in shedding interval length variance between time periods, with no significant change in variance for most animals (Fig. 3B, Brown-Forsythe test, Table S3), and three decreasing variance over time (animal D, F, J; Fig. 3B, Table S3). Concomitantly, the waveform patterns of shedding are still recognizable but are spaced slightly farther apart in the late period due to a lengthened shedding interval (Fig. 2C, D). Thus, there is no evidence supporting an accumulation of noise in the system of tooth replacement.

The mean period of tooth turnover increases between periods and scales with somatic growth

During the early period, a shedding interval was 23.1 days long on average. In the late period, the average shedding interval was 31.2 days, demonstrating that the tooth cycle lengthens with age ($p < 0.001$, paired t-test, $df = 9$, Fig. 3B).

If the same biological factors affecting growth rates are affecting the rate of tooth cycling, the duration of a developmental event (e.g. a tooth shedding interval) is predicted to increase and scale as a $\frac{1}{4}$ power law to body mass, or a $\frac{1}{4}$ slope in a log-log plot (Lindstedt and Calder III 1981; Glazier 2005). During the full study period, the geckos are juveniles and weight gain is essentially linear (Fig. S2A), although individual rankings by weight shift over the two observation periods (Fig. S2B). Within our dataset, we see that the cohort-level log-log slope over the study period is very close to 0.25 and its 95% confidence interval contains $\frac{1}{4}$ (slope of 0.2354 ± 0.1192 , 95% CI, Fig. 3C), suggesting the $\frac{1}{4}$ power law holds over the study period. Although eight animals follow this relationship as they grow, two do not (animals H, I; Fig. 3D), with their individual slopes falling outside of the 95% confidence interval for the cohort slope. In this sample, inter-individual differences in tooth turnover during an observation period do not scale with a linear slope to their differences in weight (within-group scatter, Fig. 3C).

Scaling at the cohort level suggests that metabolic processes related to growth may be driving the increase in shedding interval, but individual variation in these processes does not appear to explain inter-individual differences in tooth turnover at either time period. Moreover, the animals that do not scale their shedding intervals to the $\frac{1}{4}$ power with growth suggest that there are factors intrinsic to the dentition that affect tooth shedding clocks and may be able to decouple physiological growth processes from developmental ones.

Two motifs of shedding asymmetry persist between periods

The measurement of symmetry in shedding is a test of the genetic contribution to postnatal tooth replacement patterns. In addition, evaluating symmetry allows for the detection of small deviations from the overall shedding pattern that could not be appreciated by looking at individual jaw quadrants alone. Any micropatterns in these deviations among teeth can be evaluated for regionality and stability over time. In order to accomplish these analyses, individual tooth positions were mapped (Fig. S1) and then the right side data were overlaid on top of the left side (Fig. 4A, B) to obtain a graphical readout of symmetry.

In seven animals (A–G) we observed a characteristic time lag motif in shedding events throughout the jaw (Fig. 4A, B, Fig. S3). A tooth position showed exfoliation first on one side of the jaw and the next wax impression captured the shedding of the contralateral tooth (within 3–4 days). In contrast, the remaining three animals (H–J) displayed a position-shifted pattern. For example, one side of the dentition shed a tooth at position number 20 while simultaneously the opposite side shed a tooth at position 21 (Fig. 4C, D, Fig. S4). This position shifting was present as early as the first wax impression.

Strikingly, these qualitative asymmetry motifs were maintained between early and late periods (Fig. 4A–D) in the posterior maxilla. Between symmetrical shedding events and these fine-scaled departures from symmetry, shedding events remain overwhelmingly pairable between right and left sides throughout the study period.

Although genetic and physiological conditions appear to supersede the effects of environmental perturbation in the late period gecko dentition, it is still unclear to what extent these biological effects act on individual tooth germs versus the entire dentition. To evaluate whether or not teeth are individually renewing units, we studied the asymmetry of shedding events across the dentition in more detail.

Persistence of symmetry in shedding patterns post-hatching

There was a trend of increasing asymmetry in tooth shedding from the premaxilla to the posterior maxilla in all 10 animals (Fig. 4E). The three animals with a frame-shifted asymmetry motif had a higher baseline level of asymmetry than the rest (H, I, J). Two of these animals are also the same ones that showed divergent scaling of their shedding intervals with animal growth (Fig. 3D). This suggests that factors local to the dentition may be acting more strongly in these individuals. To partition any shared factors that might be more significantly affecting dental symmetry in these three individuals as compared to the remaining seven with the more expected asymmetry motif and predicted scaling relationships, they were analyzed in a distinct group from the others. We tested whether the observational period, jaw region and/or shedding motif had significant explanatory power on

percent asymmetry within each individual. Of these factors, jaw region and motif were each statistically significant in explaining the organization of gecko tooth shedding asymmetry (Table 2). Post-hoc pairwise testing confirmed that the premaxilla was always significantly more symmetric than the posterior maxilla with the anterior maxilla as a transition region, indicating that position in the jaw affects whether tooth shedding tends to be symmetric or not (Fig. 4F, Table S4).

Percent asymmetry values were consistent between observational periods (Fig. 4F). Thus, along with shedding interval length, the overall levels of asymmetric shedding represent a second jaw-wide phenotype that does not support a significant influence of the environment on turnover of the post-hatching gecko dentition.

The categorization of individuals between motifs explains a significant portion of the variation in percent asymmetry within the dentition (Table 2). The three individuals with the frame-shifted motif had significantly increased asymmetry from the earliest shedding events recorded. In other words, these animals were affected within the dentition patterned pre-hatching as well as in newly initiated teeth post-hatching.

Directional bias in asymmetry reveals synchrony between tooth positions

Since tooth position in the jaw is predictive of how coordinated bilateral tooth replacement is, it provides evidence that, at least at one point in development, tooth positions were not independently patterned. We wanted to understand if this was a one-time constraint set up in the egg or whether a more active mechanism maintains synchrony in tooth cycling after hatching. We focused on the 7 animals with a time lag motif since this was the more representative pattern.

We redefined shedding events as bilateral events that could be classified as symmetric, isolated asymmetric, and polarized asymmetric (Fig. 5A). From a total of 664 such events, we compiled an average of 47.4 events per observational period per individual in the posterior maxilla, where asymmetries were most pronounced.

Individual variation was quantified by the number of polarized bilateral events in each array. Regardless of how strongly the time lag motif is expressed in the early period, polarized bilateral events increase into the late period in 6/7 cases, approaching a majority of events concurrently with the lengthening of shedding intervals and tooth absence durations (Fig. S5).

If each shedding event is independent of the others, it should be common to observe randomness in the polarity of bilateral shedding events because there is an equal chance of the tooth position on either side shedding first. Furthermore, when examining these polarized bilateral events, it should be common to observe flipping in the polarity of bilateral shedding events between tooth positions and time points.

We classified polarized bilateral shedding events by which side of the jaw leads the event (Fig. 5B). For each individual and time period, we modeled a binomial process corresponding to the observed number of polarized events, and evaluated the posterior maxilla of each array for right-left polarity bias. During the early period, 5/7 arrays were

found to have significant directional bias in the polarity of their time lag events, indicating that bilateral shedding events were synchronized between tooth positions and successional teeth. This suggests that the polarity observed in shedding asymmetry cannot be attributed to the stochastic component of shedding of synchronized teeth, and the time lag between contralateral shedding events within the jaw may be patterned *in ovo*.

In the late period, 6/7 individuals show a polarity bias in the posterior maxilla. The direction of polarity is maintained in all four of the animals for which both periods showed bias (Animals A, D, F, G, Fig. 5B). Thus, in the majority of animals, shedding asymmetries set up *in ovo* are propagated into later life. It is, however, possible to lose (Animal E) or gain (Animals B, C) this bias over time. Five individuals (animals A, B, C, D, F) are biased to the right side, and two show a bias to the left (animals E, G).

An array that returned a nonsignificant bias may still have biased polarity in subregions of the jaw. In the example arrays presented (Animal B), right side-biased events occur between tooth positions 9 and 18 whereas the left side dominates events in positions 21–30 (Fig. 4A). At the later time period, the entire posterior maxilla consists of right-biased events while anterior to position 9 the time lag events are left-biased (Fig. 4B). This regionalized flipping of shedding polarity is also detectable in two other individuals with differing regional boundaries (D early and late, E late, Fig. S3). Polarity of shedding may not be propagated entirely faithfully over time. Instead, contiguous tooth positions may be synchronous in their change and maintain that state for some time (at least 8 weeks).

DISCUSSION

Our study has shown that wax bites accurately record tooth exfoliation and that turnover is rapid. Within a relatively short period of six months, patterns can be observed that represent the regulation of the tooth replacement cycle. Through the comparison of two time periods, one in which the functional teeth were patterned symmetrically *in ovo* and a second period where teeth were initiated post hatching, we were able to infer both genetic and environmental factors influencing tooth replacement. These data have revealed bi-level synchrony within the jaw: retention of an overall shedding framework and asymmetry motifs that are propagated within that framework.

Deviations from right-left symmetry were a sensitive way to assess changes across jaw regions and time. The degree of perfect symmetry in our study geckos (56.1% on average) is slightly lower than the 61–87% symmetry of absence in functional teeth noted in fixed specimens of lab-reared *Chalcides varanus* (Delgado et al. 2003). Most other previous studies mention reptile dental symmetry in terms of differences in tooth counts per bone on the right and left sides (Bauer and Russell 1990; Greer 1991; Rasmussen 1996; Torres-Carvajal 2002; Hosono et al. 2007; Brown et al. 2015). In contrast, by tracking tooth positions over time with dense temporal sampling, we uncovered asymmetry motifs within previously recognized shedding patterns. Time lagged shedding events are evidence that bilateral shedding remains near-synchronous long after hatching, occurring at contralateral positions within a time period of 3–4 days out of a total shedding interval of close to 30 days. The persistence of these asymmetry motifs means that tooth cycling is not very susceptible to

environmental or metabolic perturbations, counter to predictions from both the noise accumulation hypothesis (Hallgrímsson 1993) and biological process noise scaling hypotheses (Lindstedt and Calder III 1981; Soule 1982). Two caveats are that there is certainly less environmental variability in lab-reared animals than in the wild, and we cannot rule out greater environmental influences later in life. Leopard geckos are known to live in excess of 10 years in captivity (de Vosjoli et al. 2005) and our study ends at 8 months of age.

The time dimension of shedding considerably deepens our insights into dentition renewal by capturing long-term changes to the shedding framework over the entire jaw. The length of time between exfoliation of teeth increases by the predicted $\frac{1}{4}$ power law with somatic growth in animals that follow the typical shedding pattern, a time-lagged asymmetry motif within the wave replacement scheme. An increase in body mass as seen with growth is thought to require a change in overall maintenance metabolism due to the physical constraints of transporting resources to more distal locations in the animal (Peters 1986; Savage et al. 2004; Glazier 2005). This suggests that there are somatic factors that influence the duration of the tooth replacement cycle in the juvenile leopard gecko. An alternative manifestation of scaling may be that larger animals have a deeper dental lamina and teeth in older animals take longer to traverse the larger distance from their initiation site to eruption.

Our findings align with previous longitudinal studies in the slow worm (Cooper 1966), juvenile green iguana (Kline and Cullum 1984), and alligator (Edmund 1962) that report a slowing of replacement over time. In these studies, animals were tracked for at least two years but their ages were not reported. Ours is the first study to examine hatchling animals and associate a slowing of replacement with teeth initiated post-hatching. Furthermore, replacing teeth emerge within one week of the predecessor being shed in our animal model. This narrow window means the timing of exfoliation in the leopard gecko is tightly linked to a late stage of development of the successor tooth. This is in contrast to other taxa such as the alligator, slow-worm, and iguana in which teeth take several weeks to emerge (Edmund 1962; Cooper 1966; Kline and Cullum 1984) and the stage of completion of the successional tooth was not as predictive of exfoliation.

The low variance of the shedding interval in individual leopard geckos aligns with the key parameter used in historical models of tooth renewal, which is that each tooth position has a common rate of tooth development (Edmund 1960; Osborn 1971). Our study is the first empirical validation of this parameter and the first to show it scales with growth. Under Edmund's Zahnreihen theory (1960, 1969), this parameter corresponds to a dental lamina whose cells are constantly migrating upward and adding to itself aborally (presumably by proliferation) as an anterior-to-posterior traveling stimulus initiates teeth on the aboral boundary. Osborn's tooth family theory (1971) takes the parameter as a rate intrinsic to tooth morphogenesis and maintained by spheres of inhibitory factors. Both of these models are testable with future experiments now that a baseline is established here in the leopard gecko.

***In ovo* dentition symmetry is maintained post hatching**

Maintenance of small fluctuations around the regular shedding framework, i.e. the time- and space-shifted asymmetry motifs, reveals consistency between the pre-hatching and post-hatching dentition. Once a pattern is established in the pre-hatching period, even a divergent

pattern, it persists even after the animal has been functioning in its environment. These findings suggest that manifestations of asymmetry in shedding are due to robust processes specific to the dentition. Continuity of the shedding framework suggests that developmental memory of global pre-hatching patterning mechanisms may still be acting at tooth positions post-hatching.

Propagation of shedding patterns in teeth initiated after hatching argues strongly for an association between tooth position and individual shedding events in the jaw. The retention of perfect symmetry was localized within the jaw to the premaxilla. In a broad taxonomic survey of skeletonized museum specimens, Edmund (1969) rarely found symmetry beyond the anterior teeth, but our results in the leopard gecko show that posterior tooth shedding remains nearly symmetrical over time. Adult female slow worms ($N = 3$) had symmetrical tooth eruption in antimeric and occluding quadrants, but no spatial details were provided (Cooper 1966). Several other authors indicated some interruption of shedding waves or symmetry at the premaxilla/maxilla boundary in extinct sauropterygian specimens by comparing the relative sizes of replacing teeth (Shang 2007; Sassoon, Foffa, and Marek 2015). Together, these studies suggest that there may be stronger epigenetic or pleiotropic effects at the back of the jaw as compared to the front. Our longitudinal data illustrate the importance of *in ovo* conditions on creating the complexity of patterns in the post-hatching dentition. For example, the reliable increase in asymmetry in the posterior jaw may also be relative to the amount of space available along the bone shelf to initiate and grow teeth. Thus, the ability of the developmental system to weaken and ultimately decouple premaxillary and maxillary tooth synchrony may be an ancient property of the vertebrate dentition that has enabled its evolutionary modification into various forms of heterodonty.

Bilateral variation in tooth positions may occur pre- or post-hatching

The relatively large sample size in our study provided the power to detect the rarer tooth shedding patterns (regional and position shifted variants). Inter-individual variation in adherence to the asymmetry motifs suggest that the tooth development mechanism is the same between individuals, but that cycles at each tooth position were not synchronized to the same degree in each animal. This could be due to genetic differences (including inbreeding), or environmental stress during early development. The presence of a divergent, position-shifted asymmetry motif in a few individuals during the early period suggests that modifications to symmetrical initiation and cycling occurred *in ovo* in these individuals. These individuals also showed abnormalities in developmental processes scaling with growth. We hypothesize that a decoupling of growth and development, as well as increased departures from symmetrical patterning, could arise from an overriding of conditions within the dentition to compensate for an early developmental error in tooth patterning.

Alternatively, a position-shifted motif could arise from an interstitial tooth position added to one side of the dentition in the initial month of growth, when teeth are not sufficiently erupted to capture in wax impressions. Failure of a rudimentary tooth to initiate a replacement program could remove a tooth position and create a similar motif. We plan to test this hypothesis from hatching in other individuals by counting tooth numbers in serial photographs. We detected only one instance of a tooth position lost from the jaw post-

hatching, and it resulted in a switch of asymmetry motif from position shift to time lag (Animal A, Fig. S3). The lack of other motif switches suggests that during the course of a typical juvenile period, the number of tooth positions is consistent except for addition at the back of the jaw. Alternatively, and considerably less likely, the turnover of all posterior teeth shifts when a tooth is added interstitially to remain in line with the previously established shedding pattern.

Mechanisms producing non-independence

The ontogeny of asymmetry in phenotypes has previously been used to distinguish between developmental mechanisms present in bird somatic growth and feather growth. These studies tested up to seven hypothetical mechanisms and found the best support for noise accumulation and compensatory mechanisms to explain the observed increased randomness and decreases in asymmetry post-hatching (Swaddle and Witter 1997; Kellner and Alford 2003). The logic can be applied to the finer patterns of tooth shedding synchrony to identify novel post-hatching mechanisms in the leopard gecko.

At the fine scale of bilateral shedding event polarity, timing differences between right and left sides are resistant to change in the leopard gecko dentition. Systematic relationships between right and left sides point either to an actively synchronized jaw, or to replacing teeth with intrinsically constrained timing mechanisms. When timing does change relative to the contralateral tooth position, it often changes regionally. While the localized nature of the changes in timing argues against globally synchronized mechanisms, the synchrony of polarity change across nearly one quarter of the jaw (up to 10 tooth positions) argues against total independence of post-hatching tooth positions.

The acquisition of novel patterns of synchrony among groups of tooth positions in the later time period is evidence for an active local synchronizing mechanism or a novel intrinsic timing mechanism in juvenile animals (Swaddle and Witter 1997). On the time scale of this study, we do not see the diminution of asymmetry patterns that would suggest compensatory feedback to correct asynchrony between right and left jaw halves (Swaddle and Witter 1997; Kellner and Alford 2003). The jaw halves are unlikely to communicate directly, much like growth of fingernails on left and right hands. Instead, locally acting mechanisms could be correcting for asynchronies within a side, such as maintaining waveforms or cycle lengths.

Jaw-wide tooth development is controlled by processes that retain some capacity for long-term, coordinated change between tooth generations into adulthood. Competing historical models for tooth replacement make fundamental assumptions about the independence or coordination between tooth positions post-hatching. The Zahnreihen model postulates that tooth positions are coordinated from embryonic patterning through adulthood via the propagation of diffusible signals along the jaw (Edmund 1960), whereas other models postulate that individual tooth positions containing functional and successional teeth are the developmentally controlled unit (Osborn 1970; Osborn 1998).

Regionalized changes to shedding symmetry affect both “even” and “odd” tooth positions, which are cycling with a dramatic phase shift relative to each other (Fig. S3). In the gecko dentition, teeth are connected to a continuous dental lamina (Handrigan et al. 2010), so the

synchrony of adjacent tooth positions supports the presence of a developmental mechanism capable of creating the novel phenotypic patterns via regional, semi-permanent changes to dental lamina niche conditions rather than via direct communication between developing teeth in the same family. A similar mechanism has been proposed for the pattern seen in the length of bird feathers between molts. In that system there is incremental daily growth of bilaterally symmetrical feathers, and after a molt, the feather that had previously been longer tended to be longer again (Møller 1996). The identification of locally acting mechanisms in a post-hatching reptile identifies a previously unknown proximate mechanism by which animals can plastically change their dentitions in response to ongoing life conditions, and which may be able to respond to selective pressure to change the character of the dentition regionally.

A final possibility is that consistent environmental inputs or behaviors are responsible for consistent patterns of asymmetry. For example, the mechanical forces of catching mealworms, often experienced by the snout, may have contributed to the consistent symmetry observed in the anterior-most dentition. To counter this, we reiterate that injuries severe enough to disrupt tooth presence/absence data in wax impressions ($N = 16$) are all on the right side as a result of the right-handed investigators' technique, and no other sided bias in shedding was found despite investigator handedness. Despite these occasional disruptions, asymmetry motifs are maintained in these individuals before and after injury, arguing against mechanical force-induced tooth shedding as the main cause of tooth position synchrony.

CONCLUSIONS

Contralateral teeth have been regarded as operationally identical for the study of biological principles by most developmental biologists, anthropologists, and paleontologists. Our study in the leopard gecko shows that embryonic symmetry can be maintained for quite some time despite high turnover of individual organs making up the dentition. However, normal cycling patterns do deviate in asymmetric ways that are not corrected, suggesting that right and left sides are unlikely to be actively coordinated post-hatching.

This study provides a critical foundation for future experiments targeting the response to disruption with regards to tooth position communication. Future investigations will involve systematic perturbations to developing teeth to identify these critical periods and locations of communication as well as monitoring of compensatory dynamics in shedding arrays. Due to the apparent linkage of shedding to the symmetry of *in ovo* patterning, the effects of early development may be inferred far into life in many cases with non-invasive methods. Given the widespread detection of similar shedding patterns across reptiles, it may be possible to infer events from early development and the presence of environmental and metabolic perturbations to cycling in taxa for which we cannot observe them, such as in museum specimens or extinct taxa. Furthermore, our data suggest that *in ovo* patterning mechanisms as well as those for individual organogenesis may be retained in the dental tissues postnatally.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MicroCT isosurface of a hatched gecko maxilla, palatal view. Each tooth ‘family’ is perpendicular to the length of the jaw (blue rows of teeth, 1–7). There are equivalent families on the left. Gradients of developmental maturity termed Zahnreihen (Woerdeman 1921) are also symmetrically arranged (pink and green, a–c). The Zahnreihen are unerupted and only visible on a radiographic image. Marginal teeth are erupted. Inset: Left maxilla close-up, labels as in black boxed area of larger image. Black outlines highlight two erupted tooth positions with slightly differing stages of successional teeth. Key: mx = maxillary bone, pmx = premaxilla, rt = rudimentary tooth.

Figure 2.

Tooth shedding data collection. A) Tooth shedding study design. Tooth shedding data were collected during two 8-week periods designated in brackets. Schematics of three clustered teeth indicate tooth family composition at these time points, with a different pattern marking cycling for each tooth generation. B) Tooth replacement cycle parameters. A single tooth family consisting of a functional tooth (generation n) and its successor (generation $n+1$) is represented with developmental time progressing to the right. (a) is a tooth replacement cycle from initiation to shedding of a single tooth. (b) is the duration of a tooth absence from shedding of the first tooth to eruption of its successor, measured in number of consecutive absences at the tooth position in wax impressions. (c) is the shedding interval, the amount of time between the shedding of a functional tooth and the shedding of its successor tooth, which represents a portion of the replacement cycle. (d) small black boxes represent three shedding events, or individual missing teeth recorded in wax impressions. Overall tooth position occupancy is recorded through time as a series of white (tooth present) and black (tooth absent) boxes. C) and D) Representative tooth shedding arrays during early (C) and late (D) time periods. Columns 1–30 represent individual marginal tooth positions, with M denoting the midline tooth. Rows represent data from single wax impressions on specific dates ($N = 16$ biweekly). Black boxes record a tooth absence, or shedding event as defined above, and positions with white boxes contain a functional tooth. Posterior to anterior waveforms linking shedding events at even tooth positions alternate in time with waveforms linking odd tooth positions, as previously observed in reptiles. pmx = premaxilla, ant mx = anterior maxilla, post mx = posterior maxilla. E) Maintenance of wax impression

registration over time. A consensus configuration for Animal A during the early observation period, marking the unpaired midline tooth (red), premaxillary teeth (purple dots), and maxillary teeth (green dots). Premaxillary teeth are separated from the maxillary teeth by a diastema (white arrowhead). E') Wax impression showing individual tooth positions present (white punctate areas) and absent (black arrowheads). E'') Wax impression from E' at higher power, with gaps correctly identified as unoccupied tooth positions (black arrowheads) and gaps that do not represent missing teeth, such as the diastema between the premaxillary and maxillary teeth (white arrowhead). Scale bar = 2mm. This provides an example of how, once constructed, the consensus arch could be superimposed, rotated, and translated to bring impressions into common registration and maintain the proper identity of tooth positions over time.

Figure 3.

Properties of repetitive tooth shedding. A) Durations of tooth absences pooled across all study animals (N = 10; 1103 absences in early period, 910 in the late period). B) Bean plot of variation in shedding interval, the length of time between two shedding events at a given tooth position (animals A–J), during early (black) and late (grey) periods. Thick black lines represent shedding interval means for a given shedding array. Light blue lines are proportional to the number of times that particular length was sampled in that array, with number of scored shedding intervals in brackets below (N_{early}, N_{late}). Because wax impressions are spaced apart by several days, the length of the shedding interval is quantized rather than continuously distributed. Study-wide mean values are significantly different between early and late periods (green lines, shading SD, *** indicates $p < 0.001$). C) Individual mean animal weight for early (left cluster) and late (right cluster) observation periods is plotted against the mean shedding interval during that period. Linear regression across this dataset extracts a slope, or exponential scaling factor, of 0.2354. D) Comparison of individual ontogenetic scaling factors to the dataset average (thick black line, grey shading 95% CI) and the theoretical scaling factor of 0.25 (thick dotted line). Individuals H and I fall outside the 95% confidence interval for scaling over time.

Figure 4.

Asymmetry of shedding patterns. A–B, C–D) Representative tooth shedding arrays displaying the two asymmetry motifs, time lag (A,B) and position shift (C,D). Shedding arrays for right and left sides of the jaw are overlaid, with M denoting the midline tooth. Black boxes indicate bilateral symmetry of shedding events. Moving posteriorly in the jaw reveals more blue and red boxes indicating shedding on the left only and right only, respectively. A,B) Vertical stacks of red on blue or blue on red boxes represent the time lag asymmetry motif (green paired arrows, examples). Pink paired arrows mark time lagged events of longer duration, seen more frequently in the later period when absences are longer. C,D) Red and blue boxes are frequently adjacent to each other in neighboring tooth positions, a position-shifted asymmetry motif with elevated baseline asymmetry (green paired arrows, examples). E) Percent asymmetry values across 30 teeth of each animal in the study, averaged across time periods ($N = 10$). F) Mean percent asymmetry plotted by jaw region for each category used in the 3-way mixed ANOVA, $\pm$ SE. Two groupings representing asymmetry motifs are statistically distinguishable, but early and late observational periods are not (Table S4).

Figure 5.

Scoring of bilateral shedding event asymmetry over time and space. A) Asymmetric events classified into subtypes across the posterior maxilla in individuals (N=7) with a time lag motif. Below the graph are illustrative examples of each subtype as scored from shedding arrays. Black boxes represent symmetrical tooth absences, red boxes are right-sided absences, and blue boxes are left-sided absences. On average, polarized events accumulate between early (black bars) and late (grey bars) time periods but these trends are not significant using t-tests. Error bars represent 1 standard error of the mean percentage of all the animals. On average, the number of polarized events per animal was 15 for the early period and 22 for the late period. The number of isolated events per animal was 19 for the early period and 14 for the late period. The number of unclassifiable events was 1 for the early period and 0.5 for the late period. B) Test for polarity bias using a binomial model. Counts of polarized shedding events (n, column 1) and which side of the mouth shed first were modeled as a binomial process with an event probability of 0.5 as to which side sheds first. Bold p values are less than 0.05 and considered significant departures from the

binomial assumptions. Colored capital letters denote the directionality of the bias for each array.

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Table 1

Average recorded tooth absences per 8-week period

Region	N _{both sides} (SD)	% difference R-L (SD)
premaxilla	37.1 (6.5)	-1.6 (7.6)
anterior maxilla	31.5 (4.6)	2.2 (8.6)
posterior maxilla	112.3 (16.4)	2.6 (6.2)
<i>total</i>	<i>180.9 (23.2)</i>	<i>1.7 (4.3)</i>

Table 2

Explanatory influences on tooth shedding asymmetry based on 3-way mixed ANOVA

	SS	DF	MS	F	p value
Intercept	169726.4	1	167926.4	146.3569	0.000002
Motif	20382.8	1	20382.8	17.5763	0.003028
Error	9277.4	8	1159.7		
Observational period	4.3	1	4.3	0.0100	0.922977
Observational period*Motif	253.6	1	253.6	0.5911	0.464102
Error	3433.2	8	429.1		
Jaw region	10995.9	2	5497.9	27.1481	0.000007
Jaw region*Motif	969.7	2	484.9	2.3942	0.123134
Error	3240.3	16	202.5		
Observational period*Jaw region	338.2	2	169.1	1.0958	0.358103
Observational period*Jaw region*Motif	221.9	2	110.9	0.7189	0.502391
Error	2469.3	16	154.3		

Key: SS = sum of squares, DF = degrees of freedom, MS = mean square error, F = F statistic, p = p value. Bold rows indicate factors with $p < 0.01$.