Project *Varanus salvatorii*

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- Abstract

Reporting on the first known case of genetically proven parthenogenesis in Asian Water Monitor, *Varanus salvator*. Additionally, documenting attempts to hybridize species in Genus *Varanus* backed up with DNA results. By using the same DNA testing method, offspring are able to be tested to prove if they are the result of parthenogenesis or hybridization. More detailed background information and test results included.

- Introduction

When it comes to reptiles, it seems as though there is always another stone to turn over as we are always learning new significant information about the topics such as the animals' habitat preferences, diet or physiology. For years, rumors of virgin births have circulated the reptile community in the private sector spanning across many different species. While professional facilities, such as zoos, are capable and sometimes follow through with having the means to procure data to back up the fact that these animals lack a father, rarely if ever were the supposed parthogens in private collections able to be proven by any sort of testing and instead were solely proven by personal accounts. Particularly in the Genus *Varanus* multiple accounts have been reported in species such as Argus monitors (*Varanus p. panoptes, Varanus p. horni),* Komodo dragons (*Varanus komodoensis*), the Ornate monitor (*Varanus ornatus)* and the Water monitor (*Varanus salvator).* In this case we will be focusing on parthenogenesis of *Varanus salvator*. The case that will be reported on was the result of two female water monitors performing a pseudocopulation that resulted in both animals laying viable clutches that survived hatching.

In addition to parthenogenesis, there have been reports of hybridization within the Genus *Varanus*. However, just like the parthenogenetic offspring these accounts were not backed up by science and determined simply by the eye and testimony. The attempt was to hybridize a male Crocodile Monitor, *Varanus salvadorii*, with a wild-type female Water Monitor.

Finally, the ability to determine hybridization and parthenogenesis is now accessible in a quick and easy manner with a variety of options of samples to submit to create results that can be reported to back up the testimonies that have been floating around for years.

"Yoshi" M1 *Varanus salvator*

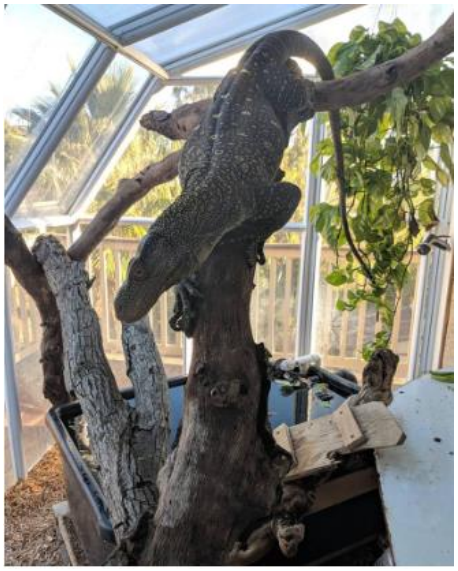
- Material and methods

Any Varanids mentioned in which DNA were gathered from to test for parthenogenesis were gathered from animals that were owned and under the care of Cory Aymar of Toothless Reptiles in Southern California at the time of breeding, oviposition and

"Blade" Melanistic *V. salvator*

hatching as applicable.

The wild-type female *V. salvator* "Yoshi" was born in 2004 and acquired in 2006. The melanistic *V. salvator* "Blade" was born in 2011 and purchased in 2016 as a male. These water monitors were each maintained in 8'x8'x8' cages with 150 gallon ponds with water at 78° F. The cages contained cypress mulch with basking areas on shelves and ground level at about 134°F through ceramic heat emitters. The ambient temperature was kept at 85°F at all times with a 14 hour light cycle. The cage also contained a lay area 48"x24"x36". The monitors were immediately introduced to each other and copulation observed. The eggs were laid and collected shortly after.



S1 Male *V. salvadorii*

The male *V. salvadorii* (S1) was acquired in December of 2017 and was kept by a previous owner for about 8 years after being field collected. S1 was kept in a natural sunroom measuring 10'x6'x8' with a cypress mulch floor. The sunroom contained large oak branches to climb and a 150 gallon pond. Humidity was maintained at approximately 70% with the assistance of a misting system. A hot spot of 88° F was maintained via ceramic heat emitters. An additional lighting system was also presented to create a 14 hour light cycle.

S1 x M1 copulation

A few months after acquiring, S1 was introduced to Yoshi in a neutral 4'x8'x4' cage. Copulation was observed only a few minutes after introduction. Three viable eggs were eventually collected.

All animals were fed a diet of primarily week old chicks with occasional rats. Females were fed twice a week as much as they would eat and males fed one large meal per week. All clutches of eggs were incubated at 82° F in Hatchrite media that is first 100% dehydrated and then rehydrated to a 1:1 ratio by weight of distilled water and media for about 7 months until hatching.

DNA samples were collected through blood or tissue and submitted to Therion Genetics in New York where DNA primer sets were established and compared.

- Results
    - Data

ID

M1 Adult Female Water Monitor (Yoshi)
M2 Parthenogenetic offspring from M1 (Yohi)
M3 Sexually produced offspring from M1
M4 Unrelated Female Water Monitor

H1 Possible Hybrid of S1xM1
H2 Possible Hybrid of S1xM1
H3 Possible Hybrid of S1xM1

S1 Male Crocodile Monitor
S2 Unrelated Female Crocodile Monitor

ID	**Primer Set 1**	
	Allele 1	Allele 2
M1	298	298
M2	298	298
M3	298	298
M4	302	302

Primer Set 1 only contains DNA from 4 water monitors – an adult female, one of her parthenogenetic offspring, one of her sexually produced offspring and an unrelated female. In this set we are able to see variation between the related and unrelated animals.

ID	Primer Set 2	
	Allele 1	Allele 2
M1	199	199
M2	199	199
M3	199	199
M4	199	199
H1	199	199
H2	199	199
H3	199	199
S1	147	168
S2	147	168

ID	Primer Set 7	
	Allele 1	Allele 2
M1	271	271
M2	271	271
M3	271	273
M4	275	279
H1	271	271
H2	271	271
H3	271	271
S1	145	153
S2	145	153

ID	Primer Set 9	
	Allele 1	Allele 2
M1	233	233
M2	233	233
M3	233	233
M4	233	233
H1	233	233
H2	233	233
H3	233	233
S1	229	232
S2	229	232

ID	Primer Set 10	
	Allele 1	Allele 2
M1	172	178
M2	172	172
M3	170	172
M4	184	186
H1	172	172
H2	172	172
H3	178	178
S1	176	208
S2	182	182

In Primer Set 2, the three potential hybrid offspring (H1, H2, H3) of S1xM1 are compared with pure crocodile monitor samples. All water monitors and hybrids in questions show the same homozygous expression while the crocodile monitors have heterozygous expression identical to each other but different from the water monitors.

In Primer Set 7, on Allele 2 of M3 we see a value not present on M1. This is explained as this is the sexually produced offspring so the variation would be from the animals' father. M4 also expresses variation but this is explained by the fact that this is an unrelated female. Note that M1, M2, H1, H2 and H3 have the same homozygous expression.

Primer Set 9 provides the same data as Primer Set 2.

In Primer Set 10, M1 has a heterozygous expression. Offspring M2, H1, and H2 have a homozygous expression identical to that of Allele 1 on M1. H3 also has a homozygous expression but from Allele 2 from M1.M3 has a heterozygous display with Allele 2 identical to Allele 1 of M1. M4 also has a heterozygous display.

ID	Primer Set 12	
	Allele 1	Allele 2
M1	338	338
M2	338	338
M3	338	338
M4	340	343
H1	338	338
H2	338	338
H3	338	338
S1	187	187
S2	187	187

ID	Primer Set 21	
	Allele 1	Allele 2
M1	264	282
M2	282	282
M3	278	282
M4	291	297
H1	264	264
H2	264	264
H3	282	282
S1	249	249
S2	251	259

ID	Prime Set 22	
	Allele 1	Allele 2
M1	193	195
M2	195	195
M3	195	199
M4	185	203
H1	195	195
H2	195	195
H3	195	195
S1	183	183
S2	201	206

ID	Primer Set 24	
	Allele 1	Allele 2
M1	176	176
M2	176	176
M3	162	176
M4	131	131
H1	176	176
H2	176	176
H3	176	176

In Primer Set 12 M1, M2, M3, H1, H2 and H3 also display the same homozygous value. M4 displays a heterozygous value different that the other water monitors. Both S1 and S2 display identical homozygous values to each other but different from the rest of the animals.

In Primer Set 21 and 22 the data is similar to Primer Set 10 where M1 has a heterozygous value and M2, H1, H2 and H3 have a homozygous identical to one of the values for M1.

Primer Set 24 presents data similar to Primer Set 12 except M4 also has a homozygous value, although different from the others.

ID	Primer Set 27	
	Allele 1	Allele 2
M1	138	138
M2	138	138
M3	138	138
M4	138	138
H1	138	138
H2	138	138
H3	138	138
S1	230	230
S2	230	230

Primer Set 27 presents all water monitors and potential hybrids with identical homozygous values. S1 and S2 also show identical homozygous values although different from the rest of the group.

ID	Primer Set 28	
	Allele 1	Allele 2
M1	332	336
M2	336	336
M3	326	336
M4	332	338
H1	336	336
H2	332	332
H3	336	336
S1	347	377
S2	347	377

In Primer Set 28, again we see that M1 is heterozygous with M2, H1, H2 and H3 homozygous to one of her alleles. M3 is heterozygous with one value not found on M1 alleles.

ID	Primer Set 33	
	Allele 1	Allele 2
M1	357	365
M2	357	357
M3	361	365
M4	361	361
H1	365	365
H2	357	357
H3	357	357

Primer Set 33 presents the same data as Primer Set 28. M4 has a homozygous display here while having a heterozygous display on Primer Set 28.

By analyzing primer sets of the mother (M1), there is complete consistency between her and her offspring "Yohi" (M2). While Yohi displays homozygous counts at all primer sets, on alleles when Yoshi displays heterozygous counts, the value Yohi displays is always one of the two displayed by Yoshi. These alleles were compared to a sexually produced offspring of Yoshi (M3) as well as an unrelated adult female (M4). As can be seen in the charts above there is variation between Yoshi and the sexually produced offspring dictating the differences of the mother and fathers genetic material that was passed down. The unrelated adult female also helps to show the data is not so broad that we are mistaking animals as being related by confirming that the testing method is able to pick up differences. Additionally, data was collected from the three potential hybrids (H1, H2, H3) as well as the male crocodile monitor (S1), who was the potential father of the hybrids, and an unrelated adult female crocodile monitor (S2). In order to prove that the offspring were hybrids and not the result of parthenogenesis there should be variation in the alleles between M1 and H1, H2, and H3. While the primer sets might not necessarily work perfectly with the crocodile monitors, the result of having a crocodile monitor father rather than no father should still be apparent.

- Discussion

At present, all known cases of parthenogenesis are in captive situations. This is easily explained by looking at the innumerable obstacles it would take to observe a monitor lizard constantly to track breeding followed by collecting DNA from parent and offspring.

After about 10 months of breeding with Yoshi and low fertility rates, Blade laid 16 eggs. At that point eggs laid by Yoshi, believed to be fathered by Blade, were still incubating. The first parthenogenetic offspring "Yohi" hatched on May 9, 2017. Interestingly a parthenogenetic clutch was also deposited from "Toothless", a melanistic female, in which only one viable egg was ever collected always expressing the same melanistic trait.

As it turned out, Yohi, the offspring produced through parthenogenesis was a male. This is consistent with what would be expected evolutionarily and genetically. For one, parthenogenesis would be a highly efficient model of populating a new territory, such as an island. If one lone

female was to swim to the island, or travel to this new territory, it would be highly advantageous to the species if she was able to asexually produce offspring. It would be even more advantageous if the offspring was male as this would lessen and eventually eliminate the need to asexually produce. The advantage here is that sexual reproduction is less costly metabolically than asexual reproduction as well as sexual reproduction should result in higher fertility. Additionally, it is widely known that Varanids have a ZW/ZZ chromosome system where females possess ZW and males ZZ. Since WW is not a viable chromosome set, the only possible way for a female to replicate her chromosomes in a homozygous form would be ZZ and produce only males. If females were able to produce female parthenogenetic offspring it would indicate a XY/XX chromosome system. Given the geographic location of water monitors, on islands or mainland with nearby islands, it should come to little surprise of how many subspecies exist. Many, if not most of these animals, likely traveled to and colonized the islands via parthenogenesis and eventually led to speciation.

Melanistic parthenogenic offspring from "Toothless"

All three of the potential hybrids put together for comparison. Top has no egg tooth, middle egg tooth points straight down and bottom egg tooth points straight out.

When it comes to deciding whether or not the possible hybrids were fathered by the crocodile monitor or produced through parthenogenesis all of the data points in the same direction. They were the result of parthenogenesis rather than hybridization. When comparing H1, H2, and H3 the data is identical to that of M2, the already proven water monitor produced through parthenogenesis, and much less genetic resemblance to M3, the sexually produced water monitor. Just like M2, the potential hybrids all have a homozygous expression at all primer sets with a value exactly the same as M1 if she was homozygous or one of the two values she displays if she was heterozygous at that primer set. There was no indication of any other genetic influence other than from the mother. Additionally, each of the potential hybrids was born with a

snout deformation, an overbite, and three differently deformed egg teeth. One had an egg tooth that pointed straight down, one pointed straight out and one was completely missing an egg tooth. All of this is consistent with the genetic bottlenecking that occurs through parthenogenesis as the genes are concentrated in an extreme form of inbreeding. This also has an effect on the fertility of the clutch which is typically much lower than that of a sexually produced clutch. This is consistent in all instances here where parthenogenic clutches only have 1-3 viable eggs. Unfortunately, one of the "possible" hybrids failed to thrive and passed away. After a necropsy it was confirmed to be a male based on the presence of testes further verifying parthenogenesis by providing the final authentication.

So why wasn't the Crocodile monitor able to successfully reproduce with Yoshi? This brings up the discussion of the barriers of hybridization.

- **Prezygotic Barriers** – before the egg is fertilized
  - **<u>Spatial Isolation</u>** – <u>Are the animals located in the same geographic location?</u>
    - Water monitor – South and Southeast Asian – India, Bangladesh, Sri Lanka, Myanmar and Thailand, Cambodia, Laos, Vietnam, the Chinese Guangxi and Hainan provinces, Malaysia, Singapore, Sunda Islands Java, Bali, Borneo and Sulawesi

Image from IUCN *Varanus salvator*, https://www.iucnredlist.org/species/178214/7499172

    - Crocodile Monitor – New Guinea

Image from IUCN *Varanus salvadorii*, https://www.iucnredlist.org/species/42485775/42485784

    - No recorded extant populations overlapping but not impossible given the water monitor ability to travel and populate new areas

        - Would inhabit similar habitats - lush vegetation, water monitor less bothered by human activity, crocodile monitor mainly arboreal

Being in captivity is the easiest way to ensure that this barrier is overcome.

- **Temporal Isolation** – Are the animals active at the same time of day? Reproductive at the same times of the year?
    - Water monitor is diurnal
    - Not enough known about crocodile monitor habits and reproduction cycle
    - If temperature dependent cycles would be the same due to geographic location
- **Behavioral Isolation** – Will they recognize each other as a mate?
    - Never a problem for males - species are also similar in size and general appearance
- **Mechanical Isolation** – "Lock and key" Are the sex organs compatible?
    - Yes - despite much confusion about this topic the lock and key mechanism is not specific enough to prevent the hemipene of a different species of monitor of a similar size from entering a female. The lock and key mechanism does not focus on minute spurts of tissue on hemipenes but rather grotesque differences in size - i.e. attempting to breed *Varanus acanthurus* with *Varanus komodoensis*. In the attempted hybridization of the Crocodile monitor and the Water monitor there was no evidence or observation of any struggle with organ size or shape.

In the captive setting described, these barriers were observed to be conquered.

- **Gametic isolation** – Will the sperm be able to fertilize the egg?

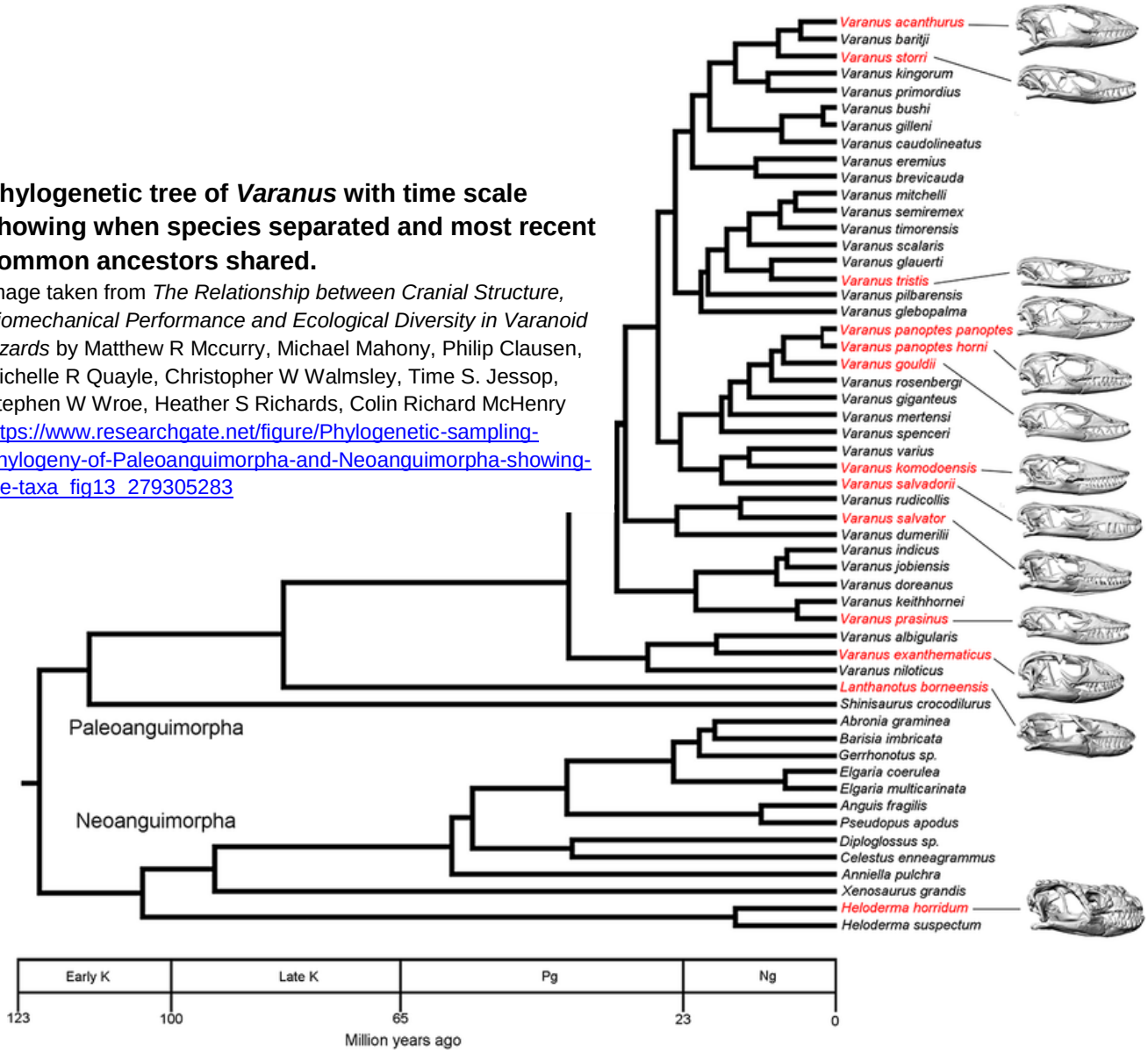
It would have been this point where the hybridization barriers were not able to be overcome, and the parthenogenesis outcompeted sexual reproduction.

- **Postzygotic Barriers –** after egg is fertilized. While we know that the process did not make it this far these barriers are still are worth mentioning.
    - **Hybrid Inviability** – Could the offspring grow to a healthy adult?
    - **Hybrid Sterility** – Would the hybrid be able to reproduce?
    - **Hybrid Breakdown** – Would the hybrid produce offspring that would be infertile or not healthy in some way?

But why wouldn't the crocodile monitor sperm be able to fertilize the egg of the water monitor despite apparently coming into contact? Given that it is possible for species in different phylogenetic Families to hybridize such as a sea urchin, *Strongylocentroltus purpuratus*, and sand dollar, *Dendraster excentricus*, it would seem that members of the same genus would be able to without issue. When referencing gametic isolation as a reproductive barrier it is most easily used to describe sessile sea creatures that release their gametes into ocean to allow them to

fertilize. Obviously, if every sperm was able to fertilize every egg we would not have any species in the ocean but instead a muddy genetic pool of sloppiness. Regardless of ocean animals or large Varanids, the isolation occurs for the same reason: lack of genetic relatedness. When observing a phylogenetic tree the distance of the line and node is relative to time. Each node represents the most recent common ancestor of all species that branch off of it. This genetic distance is how we are able to determine relatedness. Less genetic distance would correlate to species being more related than species with greater genetic distance.

Using a phylogenetic tree of *Varanus*, we are able to see the genetic distance between the water monitor and crocodile monitor. The two species shared a common ancestor approximately 40 million years ago. To put this into perspective humans and chimpanzees shared a common ancestor approximately 6 million years ago. This drastic genetic difference is likely the reason the species were not able to hybridize. The most recent ancestor of *V. salvadorii* is also shared by the most recent ancestor of *V. komodoensis* (Komodo Dragon) and *V. varius* (Lace monitor) with about 20 million years of separation. *V. salvadorii* is more closely related to the Australian monitor's than to Varanids from Asia, and least related to Varanids from Africa. *V. salvator* is



Phylogenetic tree of *Varanus* with time scale showing when species separated and most recent common ancestors shared.

Image taken from *The Relationship between Cranial Structure, Biomechanical Performance and Ecological Diversity in Varanoid Lizards* by Matthew R Mccurry, Michael Mahony, Philip Clausen, Michelle R Quayle, Christopher W Walmsley, Time S. Jessop, Stephen W Wroe, Heather S Richards, Colin Richard McHenry https://www.researchgate.net/figure/Phylogenetic-sampling-Phylogeny-of-Paleoanguimorpha-and-Neoanguimorpha-showing-the-taxa_fig13_279305283

most closely related to *V. rudicollis* (Black Roughneck Monitor) with about 18 million years of separation. To provide the highest likelihood of overcoming gametic isolation species should have the least amount of genetic distance between their common ancestors. It is accepted that speciation can take about 1 million years so with anything much longer than that the likelihood of successful reproduction begins to drop.

With this in mind, given the genetic distance of the Genus *Varanus* it would seem that there are only a handful of species that would be capable of hybridization. But keep in mind that anything could happen.

- Conclusion

Based on what we know about the water monitor's geographic location and chromosome system, it comes with little surprise that they are capable of, and frequently exhibit cases of parthenogenesis. Moving between islands that are easily inhabited would help to lead to the sub-speciation present in *V. salvator*.

When it comes to comparing the known parthenogenetic baby to the potential hybrids, the data overwhelmingly points to the fact that they are not hybrids but also products of parthenogenesis. Based on the evidence there is too much genetic distance between the two species for them to be able to still produce a viable offspring. Based on the geographic location of the water monitor and how many islands they inhabit with numerous subspecies it is not surprising that *V. salvator* is able to produce asexually. The question then comes of what could be triggering the cells to start replicating into a living creature? In other species of *Varanus*, parthenogenesis was able to be triggered by stressors such as withholding food. In this case, Cory did quite the opposite in feeding the animals as much as they wanted to eat twice a week. Instead it seems to be triggered by the copulation, or at least the attempt thereof. More information would be needed in order to draw on any measurable conclusions.

In the end, it seems that there is just too much genetic distance between *V. salvator* and *V. salvadorii* in order to produce a viable offspring. Based on the evidence, it seems that there is too much genetic distance between most species of *Varanus* to be able to produce a viable offspring due to how long it has been since they shared a common ancestor.

For questions and more information on the genetic testing described and the animals in this write up contact Cory Aymar on Instagram @ToothlessReptiles
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