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QUANTITATIVE GENETIC ANALYSIS OF REPRODUCTION TRAITS IN BALL PYTHONS

by

Benson H. Morrill

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Animal Science

Approved:

Lee F. Rickords Major Professor	Thomas D. Bunch Committee Member
Christopher J. Davies Committee Member	Justin G. Julander Committee Member
Susannah S. French Committee Member	Kenneth L. White ADVS Department Head

Mark R. McLellan
Vice President for Research and
Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

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ABSTRACT

Quantitative Genetic Analysis of Reproduction
Traits in Ball Pythons

by

Benson Howard Morrill, Doctor of Philosophy

Utah State University, 2011

Major Professor: Dr. Lee F. Rickords
Department: Animal, Dairy and Veterinary Sciences

Although the captive reproduction of non-avian reptiles has increased steadily since the 1970's, a dearth of information exists on successful management practices for large captive populations of these species. The data reported here come from a captive population of ball pythons (*Python regius*) maintained by a commercial breeding company, The Snake Keeper, Inc. (Spanish Fork, UT). Reproductive data are available for 6,480 eggs from 937 ball python clutches. The data presented suggest that proper management practices should include the use of palpation and/or ultrasound to ensure breeding occurs during the proper time of the female reproductive cycle, and that maintenance of proper humidity during the incubation of eggs is vitally important.

Ball python reproduction traits (clutch size, clutch mass, relative clutch mass, egg mass, hatch rate, egg length, egg width, hatchling mass, healthy offspring per clutch, week laid, and days of incubation) were recorded for the clutches laid during this study. For the 937 clutches, the identity of the dam and sire were known for 862 (92%) and 777

(83%) of the clutches, respectively. A multivariate model that included nine of the 11 traits listed above was compiled. Heritability and genetic and phenotypic correlations were calculated from the multivariate analysis. The trait that showed the most promise for use in artificial selection to increase reproduction rates was clutch size due to considerable genetic variation, high heritability, and favorable genetic correlations with other reproduction traits.

Although large datasets have been published for twinning in avian species, relatively few are available for non-avian reptiles. Reported here are 14 sets of twins produced from 6,480 eggs from 937 ball python clutches. The survival rate for twins during the first 3 months of life in our study was 97%. Interestingly, 11 of the sets of twins were identical in sex and phenotype, and additional genetic data suggested the rate of monozygotic twinning within this captive population of ball pythons was higher than that of dizygotic twinning. Further, using microsatellite analysis we were able to generate data that shows three sets of python twins were genetically identical.

(143 pages)

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Benson H. Morrill

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ABBREVIATIONS

μ	overall mean
AGE	age of the female at the time of oviposition
AGE_{ik}	fixed effect of the age of the ith individual in the kth year
ALL	all clutches in this study
BA	number of beneficial alleles (color/pattern) that the female is known to have, estimate of market value
BA_{i}	fixed effect of beneficial alleles of the ith individual
BP	ball python (*Python regius*)
CCL	number of consecutive years the female has laid clutches
CCL_{ik}	fixed effect of consecutive clutches for the ith individual in the kth year
CMAS	clutch mass
CP	carpet python (*Morelia spilota*)
CSIZ	clutch size
CV	coefficient of variation
EC – EE	eggs that were laid with the majority of the clutch from laid early clutches
EC	clutches with one or more eggs that were laid early
EE	eggs that were laid early
EL	average egg length per clutch excluding “slug” measurements
EL+	average egg length per clutch including “slug” measurements
EMAS	egg mass
EW	average egg width per clutch excluding “slug” measurements
EW+	average egg width per clutch including “slug” measurements
FAC	designation of which facility the breeding took place, 1 = CA, 2 = UT
FAC_{ik}	fixed effect of the facility location of the ith individual in the kth year
h^2	narrow-sense heritability, hereafter referred to simply as heritability
HMAS	hatchling mass
HOFF	number of healthy offspring per clutch
HR	hatch rate per clutch
INCD	number of days a clutch was incubated before hatching
IT	designation of incubation temperature eggs were exposed to, 1 = 31.5°C, 2 = 31.0°C
IT_{ijk}	fixed effect of incubation temperature on the clutch from the ith individual and jth sire in the kth year
MAS	mass of the female at the time of oviposition
MAS_{ik}	fixed effect of the mass of the ith individual in the kth year
MAT	maternal effects
MAT_{il}	random effect of the lth dam of the ith individual
MS	microsatellite
OC – OE	eggs that were inside the coils from outside egg clutches
OC	clutches for which one or more eggs were found outside the coils
OE	eggs found outside the coils of the female
OLS	ordinary least squares

PCR	polymerase chain reaction
PE	permanent environmental effects
PE_i	random effect of permanent environment on the ith individual
R	repeatability
RCM	relative clutch mass
REML	restricted maximum likelihood
rG	genotypic correlation
rP	phenotypic correlation
SE	standard error
TWCL	twin clutch number
V_A	additive genetic variance
V_{A1}	additive genetic variance of trait 1
$V_{A1,A2}$	additive genetic covariance of traits 1 and 2
V_{A2}	additive genetic variance of trait 2
V_I	individual variance
V_P	phenotypic variance
V_{P1}	phenotypic variance of trait 1
$V_{P1,P2}$	phenotypic covariance of traits 1 and 2
V_{P2}	phenotypic variance of trait 2
WKLD	week of the year a clutch was laid
YBN	year the female was born
YBN_i	the random effect of the year the ith individual was born
Y_{ijkl}	an observation for one of the reproduction traits
YR	the year the clutch was laid
YR_k	fixed effect of the kth year
ε_{ijklm}	random residual effect

CHAPTER 1

REVIEW OF LITERATURE

The captive-bred reptile industry has been growing consistently around the world since the 1970's (Barker and Barker, 2006; Brant, 2001; Hoover, 1998; Mattioli et al., 2006; Murphy and McCloud, 2010). As an example of the size of the industry, one facility in Florida, USA reported the production of 76,100 captive-bred reptiles, and 2,000,000 rodents marketed for the feeding of captive reptiles, in the year 2001 alone (Brant, 2001). A recent independent economic assessment by Georgetown Economic Services on the captive reptile industry in the United States of America reported that revenues in 2009 were between $1.0 billon and $1.4 billion for this industry (Andrew Wyatt, personal communication). Further, Georgetown Economic Services estimated that in 2009 13.6 million reptiles resided in 4.7 million U.S. households. For ball pythons in particular, tens of thousands are produced yearly in captivity, they are the most commonly kept python species, and among the most commonly kept snake species (Barker and Barker, 2006). Also, ball pythons with certain color and pattern mutations have been sold for upwards of $175,000 USD for a single animal (Murphy and McCloud, 2010).

Interest in python reproduction and natural history has also increased recently because of the colonization of Burmese pythons (*Python molurus bivittatus*) in the Everglades of Florida, USA (Barker, 2008; Barker and Barker, 2008a, 2008b, 2010a, 2010b, 2010c; Cox and Secor, 2007; Krysko et al., 2008; Pyron et al., 2008; Reed and Rodda, 2009). The USDA also recently carried out and published research on Burmese pythons (Avery et al., 2010).

Although the production of captive-bred reptiles has increased in recent decades, scant information exists on management practices for large production facilities, or on quantitative genetic analyses of reproduction traits. Knowledge such as this has been used to significantly increase breeding efficiencies in livestock animals for decades (reviewed in Hackmann and Spain, 2010; Harris, 1998). In addition, no studies have examined twinning at a larger-scale except in turtles. Therefore, no information exists on whether twinning leads to positive or negative overall effects on reproduction rates in any other reptile species, including pythons. Lastly, no genetic data exists to date that proves monozygotic twinning to has occurred in any non-avian reptile species.

Taxonomic History of *Python regius*

The first recorded study of ball pythons was by Albertus Seba (Seba, 1734). Seba’s works included two illustrations and a brief description of what would become known as the ball python (Seba, 1734, 1735). Fig. 1-1 is one of Seba’s illustrations of a ball python. With the use of Seba’s pictures, George Shaw gave the ball python its first official name, Boa regia in 1802 (Shaw, 1802). In 1849 the ball python was given the name Python Bellii (Gray, 1849). Pythons were separated into the family Pythonoidea (Fitzinger, 1826), then given subfamily status within the family Boidae (Boulenger, 1893) all before finally being classified under the family Pythonidae (Kluge, 1991).

Also noteworthy, in Europe the common name for P. regius is the royal python. Because of the shy, and often perceived as cowardly, nature of ball pythons, Barker and Barker (2006) mentioned essentially that this common name could frequently be regarded as a misnomer. However, they go on to hypothesize that this common name was likely given to P. regius because of the respect and ophiolatry this species incited from many of the cultures in West Africa (Bosman, 1705; Hambly, 1931; Williams, 1932).

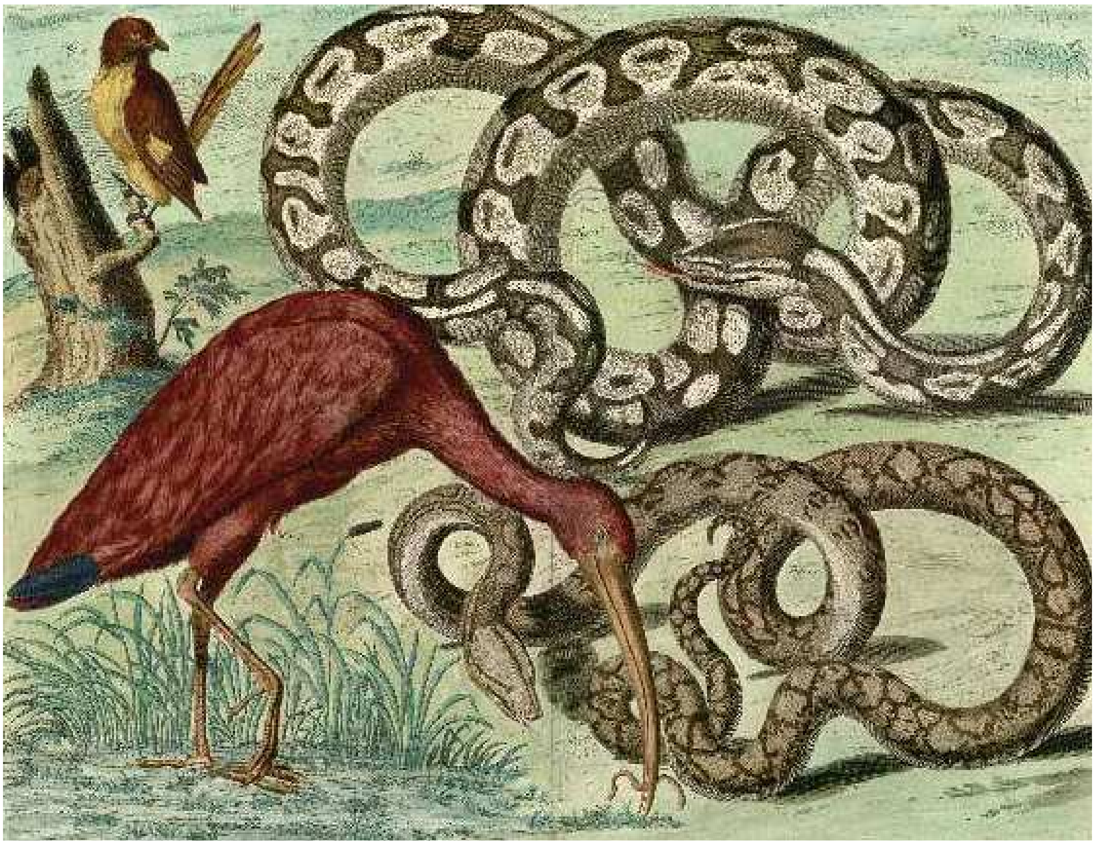

Fig. 1-1. Color illustration from the first known recorded information on the ball python from Seba (1734), plate number 62.

The relationships within the genus *Python* have not been fully resolved for several of the species, including P. regius (Douglas et al., 2010; Schleip and O'Shea, 2010). One group of researchers hypothesized that P. regius came from a common ancestor that also gave rise to the blood pythons and short-tailed pythons, (*Python breitensteini*, *Python brongersmai*, and *Python curtus*) in Asia (Underwood and Stimson, 1990). They further hypothesized that the lineage that gave rise to P. regius in Africa split and also led to the speciation of the Angolan python (*Python anchietae*). In Kluge (1991) some of the phylogenetic trees show *P. regius* and *P. anchietae* as sister species, while others list *P. curtus* as the sister species with *P. regius*. Kluge (1991) also showed all the species within the genus *Python* as well-supported independent groups, but did not distinguish the relationships within the clade. Additional details on the taxonomic history of P. regius and the family Pythonidae can be found in Barker and Barker (2006).

Ball Pythons in the Wild: Natural History and Reproduction

Ball pythons are regarded as small to medium snakes at adult snout-vent lengths that generally range from 70 cm to 170 cm, and masses that generally range from 1 kg to 4 kg (Aubret et al., 2003, 2005b, 2005c; Ellis and Chappell, 1987; Gorzula, 1998; Gorzula et al., 1997; Luiselli and Akani, 2002; Luiselli et al., 1998; Schleip and O'Shea, 2010). When encountered, ball pythons are characteristically non-confrontational and will usually coil into a ball which hides and protects the head of the python (Aubret et al., 2003; Barker and Barker, 2006; Cansdale, 1948; de Vosjoli et al., 1994). The common name, “ball python” came from the frequent use of this defensive display.

Distribution and Habitat

The distribution of ball pythons is mainly along a strip of area four to fifteen degrees N of the equator which includes north western Uganda and south western Sudan west to the coast from Liberia north to Senegal (Aubret et al., 2003, 2005c; Cansdale, 1948; Gorzula, 1998; Gorzula et al., 1997; Luiselli and Akani, 2002; Luiselli et al., 1998; Schleip and O'Shea, 2010). Although their distribution approaches the equator in Uganda, no reports have been given of any ball pythons in the southern hemisphere anywhere along their distribution.

Ball pythons are generally thought to inhabit semidesert and dry grassland areas within their range (Aubret et al., 2003, 2005b, 2005c; Barker and Barker, 2006; Gorzula et al., 1997; Luiselli, 2006; Luiselli and Angelici, 1998). However, reports have been given of them in forested areas (reviewed in Barker and Barker, 2006), and heavily altered mangrove habitat (Luiselli and Akani, 2002; Luiselli et al., 1998).

Climate and Activity

Barker and Barker (2006) summarized the climate throughout the range of ball pythons as being humid and hot during the day, and temperate and more humid at night. Daytime high temperatures frequently reach 32°C throughout the year, and during the hottest time of the year temperatures between 40°C and 45°C exist at many localities within their range. Although the light cycle and temperature are fairly stable throughout the year in this area, distinct dry and wet seasons are experienced (Luiselli and Akani, 2002; Luiselli, et al. 1998). Great detail on the climate from various localities throughout the range of ball pythons is reviewed in Barker and Barker (2006).

In their native range, ball pythons are rarely encountered above ground during daylight hours and are therefore thought to be generally nocturnal (Aubret et al., 2003, 2005b, 2005c; Gorzula et al., 1997; Greer, 1994; Luiselli, 2006; Luiselli et al., 1998; Sprawls, 1989, 1992). During the day ball pythons can frequently be found in burrows or termite mounds (Aubret et al., 2003, 2005b, 2005c; Gorzula, 1998; Gorzula et al., 1997;

Luiselli, 2006). Although ball pythons are generally regarded as being terrestrial, individuals in some populations, especially juveniles and males, have proven to be at least somewhat arboreal (Luiselli, 2006; Luiselli and Akani, 2002; Luiselli and Angelici, 1998). Higher tree-dwelling ectoparasite loads of males have been attributed to their greater utilization of trees (Luiselli, 2006).

Diet

An ontogenetic shift in diet has been shown in wild ball pythons. Individuals less than 70 cm were shown to eat mainly bird species while those over 100 cm were shown to eat mostly mammalian species (Luiselli and Angelici, 1998). This difference in diet is also seen between males and females because males tend to attain smaller adult sizes than females (Luiselli, 2006; Luiselli and Angelici, 1998). In addition, Luiselli and Angelici (1998) mention that the intersexual difference in diet is likely associated with males being more arboreal than females. When hunting, whether on the ground or in trees, ball pythons are generally thought to be ambush predators (Van Mierop and Bessette, 1981; Waas et al., 2010).

Reproductive Cycles

During the dry season, mid autumn to early spring, ball pythons are generally inactive (Aubret et al., 2003; Gorzula, 1998). From November through January pairs and small groups of ball pythons can be found together in burrows, and females are found brooding clutches from February through March (reviewed in Barker and Barker, 2006). Similar brooding behavior has been shown in python species in general (Benedict, 1932; Lourdais et al., 2007; Ross, 1977; Van Mierop and Barnard, 1976, 1978; Walsh, 1977).

Ball python females are monoestrous, therefore within the time period from February to March all reproduction for the given year takes place. With an incubation period of approximately 55-65 days, hatching of ball python eggs occurs just before the rainy season begins. The rainy season lasts from May to August (Aubret et al., 2003).

Gorzula (1998) reports the collection of 206 adult ball pythons from 24 sites. Among these were 64 adult females of which 45 (72%) were either on eggs or were obviously gravid. Female reproductive frequencies in wild python species have been reported to be less than annual in general, and as infrequent as every third year in diamond pythons (Morelia spilota) (Madsen and Shine, 1996; Slip and Shine, 1988). The high percentage of reproductive females reported by Gorzula (1988) would suggest that the reproductive frequency of wild ball pythons is less than annual, but also more frequent than biannual.

Although published data are not available on the reproductive cycle of male ball pythons, it is assumed to be similar to that of other male snakes in that a greater production of sperm, which can be observed by significantly enlarged testis, occurs in relative synchrony with the breeding season(s) (Aldridge et al., 1995; Fitch, 1970; Graham et al., 2008; Scott et al., 1995; Shine et al., 1998). Specifically, the male ball python reproductive cycle is likely similar to that of the mixed type with one period of spermiogenesis (Saint Girons, 1982). Data from reticulated pythons (Broghammerus reticulatus), blood pythons (Python brongersmai), and short-tailed pythons (Python curtus) support this assumption (Shine, 1999; Shine and Harlow, 1999; Shine et al., 1998). Also noteworthy, male ball pythons have been reported to display male-male combat during the breeding season in captivity (Schuett et al., 2001).

Reproductive Traits

Relatively little information exists on the reproductive traits of wild ball pythons. However, such reports do exist from two separate research groups (Aubret et al., 2003, 2005c; Gorzula et al., 1997). Table 1.1 summarizes the findings from these studies.

In Africa, accounts of tribes keeping pythons in what were described as fetish-houses or temples date back to the early 1700s (Bosman, 1705; M'Leod, 1820). M’Leod (1820) also mentions that the people kept the python temples swept, the pythons well fed, and that people would come to worship the captive pythons and be healed. In Europe, Albertus Seba (1665-1735) of northwestern Germany was likely among the first to keep ball pythons in captivity. Figure 1-1 depicts one of his colored illustrations of a ball python (Seba, 1734).

Ball Pythons in Captivity: History of Husbandry and Reproduction

Although ball pythons were available in the United States in the mid 1900s, they were scarce (Barker and Barker, 2006). Ball pythons were not brought into the United States in appreciable numbers until the late 1960s. By the end of the 1980s ball pythons were common in the U.S. captive reptile trade (Barker and Barker, 2006). One interesting fact about captive ball pythons is that the oldest snake ever recorded was a ball python that lived at the Philadelphia Zoo for over 47 years (Conant, 1993).

The First Successful Captive Reproduction of Ball Pythons, Logan (1973)

The earliest captive reproduction of ball pythons reported was at the Houston

Zoo from 1969 to 1972 (Logan, 1973). During this time period the Houston Zoo had three ball pythons, one male and two females. All three were purchased as wild-caught adults. The trio was kept in a cage with dimensions of approximately 120 cm long, 100 cm wide, and 140 cm tall that contained a pool of water large enough to soak in. The substrate consisted of an inch thick layer of gravel on top of concrete. Various fake plants, logs, and rocks were present in the cage as well. During the summer months the substrate temperature was generally between 26.7°C and 29°C. In the winter basking spots of 29°C were provided by two infrared lamps. The food offered to the ball pythons consisted of adult pre-killed mice.

Table 1-1
Reproductive data from two research groups on wild ball python populations. Descriptions for abbreviations are as follows: CSIZ = clutch size; MAS = post-oviposition mass of the female; CMAS = clutch mass; RCM = relative clutch mass (CMAS/MAS); EMAS = egg mass. All values are means per clutch followed by their respective standard deviations in parentheses. All values from Aubret et al. came from their 2003 paper except EMAS which came from Aubret et al., 2005c. All masses are given in grams.

Research Group Location	Gorzula et al., 1997 Ghana	Aubret et al., 2003, 2005c Togo
CSIZ	8.1 (1.7)	7.7 (1.7)
MAS	1337 (238)	1235 (241)
CMAS	772 (138)	646 (174)
RCM	0.55 (0.07)	0.52 (0.09)
EMAS	97.9 (16.3)	90.0 (10.7)

Logan noted that their ball pythons tended to spend more of their time in the cooler areas of their cage as compared to other pythons and boas at the zoo. Specifically, Logan states, “I’ve never seen our *regius* ‘bask’ under the warm spot…as do other Boids.” Logan also noted that the ball pythons began to decrease their food intake in late October or early November, and were completely off food for approximately four months of the year. Logan further stated that similar fasting among ball pythons had been reported by other U.S. zoos.

Although the male courted both of the females, only one of them was receptive and therefore all four clutches reported by Logan were laid by the same female. Clutches of eggs were laid on the following dates: March 15, 1969 (six eggs); March 11, 1970 (nine eggs); April 2, 1971 (nine eggs); and April 15, 1972 (seven eggs). In all instances the female laid the eggs during the night and the eggs were strongly adhered in a single mass when found the next morning.

Clutches were placed within plastic containers lined with moist paper towels. The eggs were covered with moist paper towels within the plastic container, and the container was sealed. The container was opened weekly and the eggs were checked to ensure they were not drying out. Two eggs, one from each of the 1969 and 1972 clutches, ruptured about two weeks after being laid and much of their albumen was lost. This was attributed to the eggs swelling during incubation and “weak areas on the shell” giving way to ruptures. In both cases the embryos developed to term and hatched, but were significantly smaller than the other hatchlings. Also, these hatchlings emerged about a week after their clutchmates.

The 1969 clutch was incubated at 26.7°C. Logan noted that several of the eggs contained weak areas on their shells. Five of the six hatchlings from the 1969 clutch emerged from their eggs on day 97 of incubation. The sixth hatchling, from a ruptured egg, hatched out on day 105.

Given the perfect hatch rate from the 1969 clutch, the eggs from the 1970 clutch were also incubated at 26.7°C. Logan noted that this clutch had the best appearance of all the clutches because very few weak areas could be seen on the shells of the eggs. However, only two of the nine eggs produced healthy hatchlings. These two eggs hatched on day 102 of incubation. The other seven eggs contained fully-formed embryos that were dead in the egg.

Worried that the low hatch rate for the 1970 clutch could have been due to an excessively long incubation period, the 1971 clutch was placed in a different incubator at a higher temperature. Unfortunately, the thermostat for this incubator was poor and the eggs reached temperatures over 37.8°C. The embryos within all nine eggs expired before hatching.

The 1972 clutch was incubated at 26.7°C. Once again, several of the eggs had weak areas on their shells, and one ruptured on day 14 of incubation. After 90 days the eggs began hatching and young emerged from 4 of the 7 eggs. On day 91 two more hatchlings were observed with their heads protruding from their eggs, as is common just prior to emergence from eggs. The following day both these young were found dead with their heads still protruding through their shells. The last egg to hatch was the one that had ruptured early in incubation. A small but healthy hatchling emerged from this egg on day 95 of incubation.

Among the various observations that Logan reported from these captive-bred clutches and offspring was that the female was seen twitching her muscles while incubating her eggs. Such shivering during incubation had been shown in *P. molurus* to increase brooding temperatures above that of the ambient temperature (Hutchison et al., 1966; Vinegar et al., 1970), and has subsequently been shown to occur in other python species (Harlow and Grigg, 1984). Also, the presence of an egg tooth was mentioned. Further, Logan reported that the egg tooth was lost before the first ecdysis. Logan also noted that the young nearly always took multiple days to complete the hatching process. During the hatching process they were frequently observed with their heads protruding through their shells, which they would usually retract defensively when disturbed.

The most commonly observed circumstance in which this pair copulated over the years was just after the female was removed from her eggs. The other frequent time at which this pair copulated was just after the female shed her skin. Logan concluded that some odor must be emitted from the female during these processes that stimulated breeding behavior in the male. The duration of such copulation periods for this pair was reported to be several days in length.

The First Successful Captive Hatching of Ball Pythons by Maternal Incubation

Van Mierop and Bessette (1981) reported the first hatching of ball pythons that had been maternally incubated in captivity. They reported on two maternally incubated clutches from two different females; one oviposited in June 1978, the other in March 1979. The female that maternally incubated the clutch in 1978 was considerably smaller than the one in 1979 (980 g versus 2010 g). The smaller female was placed in a 60 cm X 30 cm X 30 cm glass tank for the duration of the maternal incubation period. The glass aquaria contained a 3 cm layer of damp sphagnum moss on top of a 2 cm layer of damp peat moss, and a piece of driftwood was present. The larger female was placed in a 90 cm X 38 cm X 30 cm glass cage with similar interior components. Incandescent bulbs were used to control the heating within the cages. The relative humidity was maintained above 90 percent throughout the incubation periods by misting within the cages frequently.

No matings with the smaller female were observed. She became noticeably more irritable and aggressive just prior to laying her eggs. She also began to lay sideways or upside-down under the basking light during this time. She laid four eggs on June 19, 1978. The mean coil temperature for the duration of incubation was 30.6°C. Van Mierop and Bessette report observing this female leave her eggs almost daily to bask in the hot spots within the cage. Coil temperatures were elevated up to 2°C above ambient temperatures after such basking behavior. This female was offered small pre-killed rats on four occasions when she was off her eggs and she ate three of those times. After two weeks of incubation, two of the eggs began to degrade. At 63 days of incubation the other two eggs began to hatch, with emergence from the egg two days later. After their first shed the hatchlings fed on live newborn rats. After one year the young had increased in mass by six-fold.

The second pair was observed mating on several occasions. The behaviors of the second female prior to oviposition and during maternal incubation were similar to those described above for the first female. However, the second female refused to take any food items while incubating her eggs. This female laid her clutch of seven eggs on 7 May 1979. One of the eggs was excluded from her coils and was thus artificially incubated in a glass container with damp sphagnum and peat moss. The overall average coil temperature for this clutch was 30.1°C. Four days prior to hatching the eggs were no longer adherent and the female was then unable to coil around them. The eggs were then placed in an artificial incubator. After 67 days of incubation the young began the hatching process, including the one in the egg that was artificially incubated for the entire incubation period. After three additional days all seven young had emerged from their shells.

Contrary to the report by Logan (1973) of muscle twitching in a female when she was brooding her eggs, Van Mierop and Bessette (1981) did not observe any such behaviors by their females. They therefore concluded that as long as ball pythons are provided with ample temperature choices while they are maternally incubating their clutches, they are able to regulate the temperature of their eggs behaviorally by basking during the warmest portions of the day when needed.

Current Captive Husbandry and Reproduction of Ball Pythons

Multiple works provide the details currently accepted to be ideal for the captive husbandry and reproduction of ball pythons (Barker and Barker, 2006; de Vosjoli et al., 1994; McCurley, 2005; Seward et al., 2001). Although the majority of the following information comes from Seward et al. (2001) and additional personal communication with Dan and Colette Sutherland of The Snake Keeper, Inc. (Spanish Fork, UT), much of it is similar to that presented in Barker and Barker (2006), de Vosjoli et al. (1994) and McCurley (2005).

Breeder ball pythons are typically housed within rack systems with individual cages measuring approximately 81 cm L x 43 cm W x 18 cm H. Various types of chipped wood bedding are used as the substrate in the cages. Whole prey items are offered each week and water is available *ad libitum*. From March to October, the rodents offered are typically about 95 g, and during the breeding season (November to February) they are smaller (approximately 65 g). The ambient temperature of the breeding facility is controlled to prevent it from exceeding 29.5°C from March to October, and from dropping below 21°C from November to February. Throughout the year, a temperature gradient is maintained in each cage by providing a hot spot that is 32°C during the day and 29.5°C at night. Humidity is usually maintained in the breeding facility at approximately 60% year round using various types of humidifiers.

During the breeding season, females over 1500 g are placed in the cages of males over 500 g for one to two days and any observed breeding activity is recorded. Pairs are put together at regular intervals in an attempt to ensure that each female is bred at least once each month during this time. Once females are gravid, they are no longer placed with males.

Females known to be gravid are checked daily for eggs once they are 30 days past their post-ovulation shed. Eggs are removed immediately from each female, weighed as a clutch, counted, separated, weighed individually, measured (length and width), notated if they were infertile egg masses, and placed into an incubation container that is then placed in the incubation room. Each female is also weighed at this time, and a relative clutch mass (RCM) is calculated by dividing the mass of the clutch by the post-oviposition mass of the female. The ages of the sire and dam at the time of oviposition, when known, are also recorded. Also from these data, number of consecutive clutches laid by the female prior to and including each clutch is recorded.

The containers used for incubation are frequently Styrofoam shipping containers that measure approximately 28.5 cm L x 39 cm W x 18 cm H externally and are 2.4 cm thick. The medium used for incubation in these containers is a mixture of one part perlite and two parts vermiculite. Then, five parts incubation medium is mixed with one part water by volume and the container is placed in the incubation room several days prior to incubating eggs in order to allow the contents of the box to reach incubation temperatures. The top of each incubation box is covered with a 1 cm thick pane of glass. The incubation room is thermostatically controlled by a Helix DBS 1000 (Helix Control Systems, Inc, Vista, California) or similar to stay between 31°C and 31.7°C.

Data are also collected per egg on whether the egg is infertile, died during incubation, contained a fully formed embryo that was dead in the egg, embryo was live but deformed, or contained a healthy hatchling. For the eggs that hatched, the hatch date is recorded for each egg and each hatchling is weighed. After hatching, a hatch rate is calculated for each clutch.

History of Quantitative Genetic Studies on Reptile Reproduction Traits

Although published studies on reproductive data from wild populations of snake species are widely available (Brown and Shine, 2007; Farrell et al., 2009; Luiselli et al., 1996; Madsen and Shine, 1996; Slip and Shine, 1988), comparatively few publications have presented reproductive data from large populations of captive snakes over multiple years. Specifically in pythons, the studies that have been published on captive populations have generally been on small sample sizes (N < 30 clutches), and have been limited to reporting averages and ranges for reproductive traits (Barker and Barker, 2006; de Vosjoli et al., 1994; Ross and Marzec, 1990). The larger sample sizes and correlative data generated in studies on wild snake populations have provided researchers with the ability to study various aspects of reproduction such as: optimal clutch size (Aubret et al., 2003; Brown and Shine, 2007), repeatability of reproductive traits (Brown and Shine, 2007; Farrell et al., 2009; Ford and Seigel, 2006), nonlinear correlation between female size and snout-vent-length (Brown and Shine, 2007; Luiselli et al., 1996; Madsen and Shine, 1996), and female reproductive frequency (Farrell et al., 2009; Madsen and Shine, 1996; Slip and Shine, 1988).

Calculation of Heritabilities and Correlations

Even though the captive reproduction of reptiles has increased significantly over the last few decades, little research has focused on reproductive traits in any captive non-avian reptiles. Among the diminutive body of such research is a series of papers from data collected at the Janamba Croc Farm (Northern Territory, Australia) on saltwater crocodiles (*Crocodylus porosus*) in which they studied reproduction traits (Isberg et al., 2005a), age at slaughter (Isberg et al., 2005b), juvenile survival (Isberg et al., 2006a), and number of scale rows (Isberg et al., 2006b) in relation to skin production. Although sample numbers were sizeable for the reproduction traits studied (30 pairs of breeders and 190 clutches), the researchers were unable to calculate heritabilities (h^2)

because they did not know the pedigrees of their breeding adults (all but one pair were wild caught).

A select few smaller studies on wild snake populations and small captive populations have been able to calculate repeatabilities (R) and phenotypic correlations (rP), and in fewer still, genetic correlations (rG) and h^2 (Bronikowski and Arnold, 1999; Brown and Shine, 2007; Farrell et al., 2009; Ford and Seigel, 2006). Unfortunately, these studies generally relied on small sample sizes and were plagued with high error values. One trend that seems to exist in most snake species is a moderate to strong correlation between snout-vent length and age (Baron et al., 2010; Farrell et al., 2009; Ford and Seigel, 1994). Another trend that exists in reptiles in general, and specifically in snakes, is the trade-off between clutch size and egg mass (Bonnet et al., 2001; Brown and Shine, 2007; Ford and Seigel, 2006; Garner et al., 2002; Gregory and Skebo, 1998; King, 1993; Li-xin et al., 2006).

To date, it has been common practice to include measurements for all eggs in snake clutches when calculating R, h^2, rG, and rP even though it has been documented that snake clutches often contain some infertile egg masses, frequently referred to as “slugs,” that are discolored and smaller than the other eggs (Barker and Barker, 2006; Gorzula et al., 1997; Madsen and Shine, 1996; Ross and Marzec, 1990). Similarly, a recent quantitative study on porcine reproductive traits raised concerns about using average values from litters that include values from stillborns (Wittenburg et al., 2011).

Use of Restricted Maximum Likelihood in Animal Breeding Genetics

The Use of Ordinary Least Squares Versus Restricted Maximum Likelihood

The use of ordinary least squares (OLS) to estimate variance components that can be used to calculate values important in animal breeding genetics such as repeatability (R), heritability (h^2), and phenotypic correlation (rP) is relatively simple and straightforward. Historically, OLS was the main method by which researchers calculated R, h^2, and rP for their study populations (Akesson et al., 2007; Falconer and Mackay, 1996; Galton, 1889; Lynch and Walsh, 1998; Mousseau and Roff, 1987; Provine, 1971). However, several assumptions are made about data when OLS is used. These assumptions include the following: random mating; no directional or stabilizing selection on the traits being studied (natural or artificial); no linkage disequilibrium between the traits and/or factors, no epistasis between traits and/or factors; no covariances of traits and/or factors with environmental effects (Falconer and Mackay, 1996). Such strict assumptions are easily violated among many wild populations of animals, and frequently violated within captive populations (Akesson et al., 2008; Falconer and Mackay, 1996; Lynch and Walsh, 1998). Lastly, OLS is also sensitive to unbalanced datasets (Falconer and Mackay, 1996; Lynch and Walsh, 1998).

Restricted maximum likelihood (REML) provides researchers with a statistical framework by which they can study animal breeding genetics when their study populations severely violate the assumptions of OLS (Akesson et al., 2008; Falconer and Mackay, 1996; Lynch and Walsh, 1998). With REML researchers are able to utilize pedigree information from their population in order to calculate more accurate values for h2, rP, and rG. Therefore, REML is much less sensitive to unbalanced datasets, and more efficiently utilizes whatever data are available.

In recent years, REML has been widely used to study traits within wild and captive populations of animals (Akesson et al., 2008; Gilmour et al., 2009; Isberg et al., 2005a, 2005b, 2006a, 2006b; Shaat and Mäki-Tanila, 2009; Su et al., 1997; Wilson et al., 2007, 2009; Wittenburg et al., 2011). Akesson et al. (2008) specifically tested the use of OLS versus REML on a wild population of great reed warblers (*Acrocephalus arundinaceus*) to see if REML would provide superior estimates of h2 and rP. They concluded that REML produced more accurate (lower standard error) h2 and rP values than did OLS for the traits reviewed in their study.

ASReml as a Statistical Software Package for Analyzing Animal Breeding Genetics Data

Several statistical software packages are available that have been designed specifically for use in analysis of animal breeding genetics data using REML. The following are such software packages, followed by their associated websites in parentheses: ASReml (http://www.vsni.co.uk/software/asreml/); ASReml-R (http://www.vsni.co.uk/software/asreml/); DMU (http://www.dmu.agrsci.dk/); WOMBAT (http://didgeridoo.une.edu.au/km/homepage.php); and VCE (http://vce.tzv.fal.de/software). At the present all the above software packages are available for free download and use except the ASReml packages. For detailed information regarding the above software packages, including extensive tutorials on the use of ASReml, ASReml-R, and WOMBAT, see Wilson et al. (2009).

ASReml runs faster and is therefore capable of higher throughput than the other software packages. A convenient and helpful highlighter for ASReml coding is available within the freeware text-editor ConTEXT (Wilson et al., 2009). Also of importance, in ASReml researchers are able to calculate significance levels for fixed effects (Gilmour et al., 2009). Such a capability is vital to testing the importance of fixed effects on traits within studies and is a severe disadvantage to the use of the freeware programs. ASReml also allows for the independent assignment of fixed and random effects for all traits in multivariate analyses. Thus, ASReml allows for the calculation of all significant fixed and random effects independently for every trait within a multivariate model (Wilson et al., 2009). Such a complex multivariate model is frequently desired for studying animal breeding genetics in wild and captive populations.

History of Reptilian Twinning Studies

Large datasets are available on twinning rates for avian species (Byerly and Olsen, 1934; Sittmann et al., 1971; Thorogood and Ewen, 2006). Extensive datasets on twinning in non-avian reptile species are mostly limited to chelonian species (Eckert, 1990; Hildebrand, 1938; Tucker and Janzen, 1997; Yntema, 1970, 1971). Reports of twinning in the remaining non-avian reptile groups consist mainly of accounts of single occurrences of twinning (Aucone and Branham, 2005; Blomberg, 1979; Carpenter and Yoshida, 1967; Clark and Tytle, 1983; Curtis, 1950; Gudynas and Gambarotta, 1981; Hartdegen and Bayless, 1999; Mackness et al., 1998; Marion, 1980; Reese, 1906; Shaw, 1954; Shuette, 1978).

From the large turtle and bird datasets, reports have shown twinning to be reproductively disadvantageous due to low survival rates experienced by the twins. In turtles, a major contributor to the low survival rates observed was that a high percentage, estimated to be approximately 80%, of the twins found were asymmetrical and the smaller twin died in the egg (Tucker and Janzen, 1997; Yntema, 1970, 1971). Studies including 4,943 red-eared slider (*Trachemys scripta*) and over 6,000 common snapping turtle (*Chelydra serpentina*) eggs reported survival rates of twins to be between 40% and 50% (Tucker and Janzen, 1997; Yntema, 1970, 1971). Survival rates below 50% were reported in three-toed box turtle (*Terrapene carolina triunguis*) and eastern box turtle (*Terrapene carolina triunguis*) populations as well (Cohen, 1986; Messinger and Patton, 1995). Hildebrand (1938) reported finding only one set of completely separate twins from 100,000 diamond-back terrapin (*Malaclemmys centrata*) eggs, and they both died shortly after being found. From a study that consisted of approximately 40,000 leatherback sea turtle (*Dermochelys coriacea*) eggs it was concluded that all twins perished before hatching (Eckert, 1990). Likewise, studies on multiple avian species reported 0% survival rates for twinned embryos (Munro, 1965; Sittmann et al., 1971).

A higher occurrence of conjoined twinning in comparison to complete twinning has been reported in the turtle and bird literature (Byerly and Olsen, 1934; Crooks and Smith, 1958; Hildebrand, 1938; Sittmann et al., 1971; Yntema, 1970, 1971). Also, reports of twin eggs being larger than the other eggs from the same clutches have come from several reptilian taxonomic groups including the following: avian (Alley and Berry, 2002; Bassett et al., 1999); crocodilian (Blomberg, 1979); lizard (Carpenter and Yoshida, 1967; Hartdegen and Bayless, 1999); and colubrid snake (Singh and Thapliyal, 1973).

In two different turtle studies, attempts were made to investigate potential differences between twinning and non-twinning clutches and females (Eckert, 1990; Tucker and Janzen, 1997). Eckert (1990) found no differences in female size, clutch size, incubation period, or year associated with twin-bearing clutches compared to those without twins. Conversely, she did find that twin-bearing clutches had a significantly higher percent of yolked eggs than non-twinning clutches, and females that produced two or more twins in a single year were 17 times more likely to twin again the following year than by chance alone. Tucker and Janzen (1997) reported that twinning females were larger in plastron length and mass, and laid larger clutches than non-twinning females.

Studies on the effects of environmental conditions on the prevalence of developmental anomalies have shown that decreases in temperature or oxygen concentration can significantly increase twinning rates (Newman, 1923; Sittmann et al., 1971; reviewed in Hildebrand, 1938; Landauer, 1967). Newman (1923) reported specifically on how crowding of starfish eggs led to increased twinning rates presumably due to increased CO_2 and decreased O_2 levels among the eggs. The higher percent yolked eggs and larger clutch sizes that Eckert (1990) and Tucker and Janzen (1997) among twinning clutches could have led to such crowded conditions.

In mammals, the rate of monozygotic twinning is generally lower than that for dizygotic twinning (reviewed in Aston et al., 2008; Gleeson, 1994; although see Blickstein and Keith, 2007 for a notable exception). In the turtle literature more researchers have surmised that the twinning they have observed has been dizygotic (Crooks and Smith, 1958; Yntema, 1970, 1971) than those concluding observed twins were monozygotic (Hildebrand, 1938). Conversely, among snake species more researchers have concluded observed twinnings were monozygotic (Curtis, 1950; Mackness et al., 1998; Manimozhi et al., 2006) than dizygotic (Marion, 1980).

To date, no studies have reported genetic data showing monozygotic twinning to have occurred in any non-avian reptile species. However, microsatellites have been designed and tested for several genera within the family Pythonidae (Jordan et al., 2002; Taylor, 2005). Further, tested protocols are in place that could be used to extract DNA from shed skins (Fetzner, 1999) and use fragment length analysis to compare twins to each other and their parents (Jordan et al., 2002; Schuelke, 2000; Taylor, 2005). Such a study would provide an opportunity to test whether monozygotic twinning was occurring or not.

Summary

The captive breeding of reptiles has increased substantially in recent years. Although some commercial reptile breeders have reported annual captive production numbers in the tens of thousands for some reptile species, a dearth of information exists on reproduction traits and management practices for these species. Among the few such reports that have been published are studies in which either sample sizes were too small to calculate heritabilities without prohibitively large standard errors, or the pedigree information was too limited to calculate heritabilities at all. A recent study on pig quantitative genetics of reproduction traits called into question the use of measurements from stillborns in the calculation of litter averages. To date, infertile egg masses that were discolored and smaller than the fertile eggs have been included in all snake quantitative genetic studies. Finally, although studies with large sample sizes have been published on twinning rates and the effects of twinning on overall reproduction rates in turtle species, no such studies have been carried out on any other non-avian reptile taxonomic group.

Research Goals and Possible Applications of Project

First Objective

The first objective of my research was to analyze extensive reproduction data from a commercial ball python breeding company over several years and multiple generations in order to be able to identify management practices that were important for high efficiency of reproduction in ball pythons. From this analysis three main suggestions are given to those who wish to reproduce ball pythons efficiently: 1) Become proficient in techniques such as follicle palpation and ultrasound in order to assess the reproductive stages of females throughout the year; 2) Understand that the reproductive frequency of python females in captivity may be every other year, or even every third year and not to overly focus on getting females to reproduce every year; 3) Make provisions in breeding procedures to decrease the risk of subjecting eggs to desiccation at any time during incubation. Such information could also aid in the improvement of successful captive reproduction of rare and/or difficult to breed python species such as the black python (*Morelia boeleni*; Austin et al., 2010).

Second Objective

Objective two of this project was to perform quantitative genetic analysis on ball python reproduction traits from nine years of reproduction data that included 6480 eggs laid in 937 clutches. Given the superb pedigree knowledge within this population, the identity of the dam and sire were known for 862 (92%) and 777 (83%) of the clutches respectively, heritability (h^2) along with genetic (rG) and phenotypic (rP) correlations were calculated. I was also able to test whether measurements from infertile egg masses should be included or excluded from quantitative genetic analyses.

Maximization of healthy offspring per clutch (HOFF) was deemed to be the main goal in developing selection strategies. Estimates from the multivariate analysis for h^2 ranged from 0.21 to 0.60, and coefficient of variation (CV, measurement of genetic variation) ranged from 0.06 to 0.44. Although the highest CVs were for HOFF and hatch rate (HR), they were only of moderate heritability (0.24 and 0.28, respectively). While the heritability for egg mass (EMAS) was the highest of all the traits (0.60), CV for EMAS was only 0.13. Further, the rG and rP for EMASS and HOFF were -0.13 and 0.009, respectively. Therefore, although HOFF, HR, and EMAS were deemed important for use in creating selection criteria, they were not ideal due to lower heritability or genetic variation. Conversely, heritability for clutch size (CSIZ) was high (0.44), and the estimate of CV for CSIZ was among the highest for all the traits (0.26). Also, rG and rP between CLSIZE and HOFF were both 0.54. Given the above data, CSIZ appeared to be the most ideal trait to focus on when setting up selection criteria for our captive population of ball pythons.

Past researchers have suggested that egg width, and perhaps egg length, could provide an indication of the volume within the oviduct available for eggs (Ford and Seigel, 1989; Pizzatto et al., 2007). Due to the fact that snakes do oviposit some infertile masses among their clutches that are smaller (Barker and Barker, 2006; Gorzula et al., 1997; Madsen and Shine, 1996; Ross and Marzec, 1990), I hypothesized that the inclusion of the measurements for these infertile, smaller egg masses would decrease the correlations between these traits and their explanatory factors because these smaller masses would be poor indicators of oviductal space. Further, I hypothesized that averages for egg width and perhaps egg length that included measurements from infertile egg masses would lead to lower h^2 estimates. The data presented herein provide evidence that it would likely be beneficial for researchers to exclude measurements from infertile egg masses when they are calculating mean egg lengths and widths for use in developing breeding selection programs for ball pythons, and perhaps other snake species as well.

Third Objective

The final objective was to review the twinning data from the captive population of ball pythons and determine: 1) If twinning generally increases or decreases reproductive efficiencies; 2) If any reproductive traits correlate with higher occurrences of twinning; and 3) If monozygotic twinning has occurred among this, and other, study populations.

Although twinning in turtle species has been reported to decrease overall reproductive efficiencies because survival rates for twins are below 50%, the survival rate for twins in our captive population of ball pythons was 97%. As for reproductive traits that positively correlate with twinning, RCM was found to be significantly higher in twinning clutches than non-twinning clutches. Lastly, we present the first genetic data showing that monozygotic twinning has occurred in pythons.

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CHAPTER 2

EFFECTS OF CAPTIVITY ON FEMALE REPRODUCTIVE CYCLES AND EGG INCUBATION IN BALL PYTHONS (*PYTHON REGIUS*)[1]

Introduction

Although published studies on reproductive data from wild populations of snake species are widely available (Brown and Shine, 2007b; Farrell et al., 2009; Luiselli et al., 1996; Madsen and Shine, 1996; Slip and Shine, 1988), comparatively few publications have presented reproductive data from large populations of captive snakes over multiple years. Specifically in pythons, the studies that have been published on captive populations have generally been on small sample sizes (N < 30 clutches), and have been limited to reporting averages and ranges for reproductive traits (Barker and Barker, 2006; de Vosjoli et al., 1994; Ross and Marzec, 1990). The larger sample sizes and correlative data generated in studies on wild snake populations have provided researchers with the ability to study various aspects of reproduction such as: optimal clutch size (Aubret et al., 2003; Brown and Shine, 2007b), repeatability of reproductive traits (Brown and Shine, 2007b; Farrell et al., 2009; Ford and Seigel, 2006), nonlinear correlation between female size and snout-vent-length (Brown and Shine, 2007a; Luiselli et al., 1996; Madsen and Shine, 1996), and female reproductive frequency (Farrell et al., 2009; Madsen and Shine, 1996; Slip and Shine, 1988). Comparable research in captive populations could provide a foundation for the development of captive breeding

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programs with higher reproductive efficiencies. Knowledge such as this has been used to significantly increase breeding efficiencies in livestock animals for decades (reviewed in Hackmann and Spain, 2010; Harris, 1998). Moreover, an enhanced understanding of python reproductive traits and the correlations between them could increase success in reproducing endangered species, and other species that have been problematic to breed in captivity; such as the black python (*Morelia boeleni*) (Austin et al., 2010).

A commercial reptile breeding company, The Snake Keeper, Inc. (Spanish Fork, Utah) has been breeding ball pythons in captivity for over 20 years. Since 2002 they have been collecting reproductive data on their ball python breeding colony. During this time they have collected data on 5,344 eggs from 783 clutches. A review of these extensive data provides novel information about ball python reproduction and how various reproductive traits are associated with each other. Data presented in the present study on the duration of reproductive events in ball pythons are similar to data that have been published previously (Barker and Barker, 2006; de Vosjoli et al., 1994; Ross and Marzec, 1990). Novel data presented in this study supply information about age at first reproduction, frequency of female reproduction, effects of desiccation on hatch rate, and optimal clutch size. A correlation matrix for reproductive traits is also provided. These data provide a foundation for the design of future experiments, and for enhancing efficiencies of current and future breeding programs.

Materials and Methods

Adult ball pythons were housed in individual cages measuring 81 cm L x 43 cm W x 18 cm H with mesh tops within rack systems (Fig. 2-1). The substrate used in the caging was chipped aspen bedding. Water was available *ad libitum* and whole prey was offered each week. During the warm months (March to October), the rodents offered were approximately 95 g, and during the breeding season (November to February) they were approximately 65 g. The ambient temperature is controlled from March to October to prevent it from exceeding 29.5°C, and November to February from dropping below 21°C. Throughout the year, a hot spot is available in each cage that is 32°C during the day and 29.5°C at night. Humidity is maintained in the breeding facility at approximately 60% year round by a Humidifirst MP15 ultrasonic humidifier (Humidifirst, Inc., Boynton Beach, Florida).

Fig. 2-1. Rack system used to house adult ball pythons. Photo by Dan Sutherland.

From November to June, females that were over 1,500 g were placed in the cages of males that were over 500 g for one to two days and any observed breeding activity was recorded. An attempt was made to ensure that each female was bred at least once each month during this time. Once females were gravid, they were no longer placed with males. The date was also recorded for the following reproductive events when they were observed: ovulation, post ovulation shed, oviposition, and hatching.

Gravid females were checked daily for eggs once they were 30 days past their post-ovulation shed. Eggs were removed immediately from each female, weighed as a clutch, counted, separated, weighed individually, measured (length and width), and placed into an incubation box that was then placed in the incubation room. Each female was also weighed at this time, and a relative clutch mass (RCM) was calculated by dividing the mass of the clutch by the post oviposition mass of the female. The age of the sire and dam at the time of oviposition, when known, was also recorded. From these data, the age at first reproduction was recorded for all the breeders that first reproduced in 2003 or later. In addition, for each female that laid two or more clutches between 2003 and 2009, the number of years between reproductive events (inter-oviposition interval) was recorded as the female reproductive frequency.

The incubation boxes used were Styrofoam shipping containers that measured 28.5 cm L x 39 cm W x 18 cm H externally and were 2.4 cm thick. The incubation medium used in these boxes was a mixture of one part perlite and two parts vermiculite. Five parts incubation medium to one part water by volume was then mixed, and the box was placed in the incubation room several days prior to incubating eggs in order to allow the contents of the box to reach incubation temperatures. The top of each incubation box was covered with a 1 cm thick pane of glass. The incubation room was temperature controlled by a Helix DBS 1000 (Helix Control Systems, Inc, Vista, California) to stay between 31.4°C and 31.7°C from 2002–2005, and between 30.9°C and 31.1°C from 2006–2009.

Data were also collected per egg on whether the egg was infertile, died during incubation, contained a fully formed embryo that was dead in the egg, embryo was live but deformed, or contained a healthy hatchling. For the eggs that hatched, the hatch date was recorded for each egg and each hatchling was weighed. After hatching, a hatch rate was calculated for each clutch. For the calculation of average oviposition and hatch dates over the years, both oviposition date and hatch date are reported as number of weeks of the year.

GraphPad Prism 5.0 was used for all statistical analyses performed in this study. All traits were analyzed for normality and homoscedasticity and transformations were made when needed. Female mass and clutch mass were log-transformed prior to use in any statistical analyses.

Results

From 2002-2009, data were collected on 5,344 eggs from 783 ball python clutches. Novel information from these data include sire and dam age at first reproduction, dam reproductive frequency, and duration from last copulation to oviposition. A comprehensive summary of clutch, breeder, reproductive event, and egg data is presented in Table 2-1.

Table 2-1
Mean, standard error (SE), minimum, maximum, and sample sizes for the data collected.

Clutch Info	**Mean**	**SE**	**Min**	**Max**	**N**
Clutches/Year	97.88	22.73	34	192	8
Week Laid	23.90	0.19	4	52	708
Clutch Size	6.83	0.06	3	14	783
Clutch Mass (g)	604.61	6.69	91	1270	775
Female Mass (g)	1464.97	11.29	830	2874	759
RCM	0.42	0.004	0.07	0.71	758
Breeder Info (yrs)					
M Age	4.30	0.08	1	13	605
M Age at 1st Rep	2.25	0.04	1	6	354
F Age	6.08	0.09	2	18	771
F Age at 1st Rep	3.96	0.06	2	8	321
F Rep Frequency	1.97	0.05	1	6	251
Reproduction Events (days)					
Last Copulation to Oviposition	97.18	1.06	46	174	558
Ovulation to Shed	19.64	0.29	12	32	125
Shed to Clutch	31.36	0.25	15	46	321
Ovulation to Oviposition	51.52	0.48	37	78	155
Shed to Hatch	90.35	0.31	78	111	285
Oviposition to Hatch	58.87	0.07	53.25	66	582
Ovulation to Hatch	110.84	0.58	99.71	137	139
Egg Info					
Egg Length (mm)	75.71	0.24	42.08	99.8	759
Egg Width (mm)	45.39	0.14	24.15	54.4	757
Week Hatched	32.37	0.18	19	51	597
Hatchling Weight (g)	62.20	0.31	27.25	90.2	685
Infertile/Clutch	0.77	0.06	0	10	783
Egg Died/Clutch	0.35	0.03	0	7	783
Dead in Egg/Clutch	0.10	0.03	0	12	783
Deformed/Clutch	0.13	0.02	0	4	783
Healthy Offspring/Clutch	5.49	0.09	0	12	783
Hatch Rate	0.81	0.01	0	1	783

During this study, 27 clutches (3.4%) were recorded as having been found late (> 24 hrs post oviposition). The clutch mass, RCM, number of healthy offspring, hatch rate, egg length, egg width, and hatchling mass averages were compared between these 27 clutches and averages from all the clutches from this study (Table 2-2). Student's *t*-test was used on all data except hatch rate for which Mann Whitney test was used due to extreme non-normality.

Oviposition anomalies, such as exclusion of eggs from the dam's coils (Fig. 2-2) or early laying of eggs, occasionally occur during the laying season. Excluded eggs or early eggs were found in 15 (1.9%) and 7 (0.89%) of the clutches, respectively. The RCM, female mass, and clutch mass averages from clutches with one or more eggs found outside the coils of the female and those laid > 24 hrs prior to the rest of the clutch were compared to the averages from all the clutches from this study (Student's *t*-test) (Table 2-3). Fig. 2-3 presents the hatch rates calculated for: eggs that were found outside the female's coils (OE), clutches that had eggs pushed outside the coils (OC), eggs that were inside the coils from outside egg clutches (OC - OE), eggs that were laid early (EE), clutches with eggs that were laid early (EC), the eggs that were laid with the majority of the clutch from laid early clutches (EC - EE), and all clutches in this study (ALL). Statistical differences were calculated using the Mann-Whitney test due to extreme non-normality.

Table 2-2
P-values for comparisons between clutches found >24 hrs after being laid and all clutches from this study.

CMAS	RCM	HOFF	HR[a]	EL	EW	FMAS
0.003	**0.009**	**0.002**	**0.002**	0.057	**0.038**	0.409

P-values in bold were significant at the P < 0.05 level.
[a]Mann-Whitney test used due to extreme non-normality, all other P-values were calculated using the Student's t-test.

Fig. 2-2. Female ball python with her newly oviposited clutch in which one of the eggs was excluded from her coils. Photo by Dan Sutherland.

Table 2-3
P-values for reproductive traits from clutches with eggs laid early or not within the coils of the dam compared to all clutches.

Trait	Outside Coils	Laid Early
Relative Clutch Mass	**0.004**	**0.012**
Female Mass	0.3	0.199
Clutch Mass	0.179	**0.01**

P-values in bold were significant at the $P < 0.05$ level.

In order to analyze relationships between the reproductive traits measured in this study, a Pearson correlation matrix was generated (Table 2-4). Strengths of phenotypic correlations (rP) are termed as follows: 0.0 to 0.2, negligible; 0.2 to 0.4, weak; 0.4 to 0.7, moderate; 0.7 to 0.9, strong. Among the 28 correlations, 25 (89%) were significant at the $P < 0.05$ level, and 16 (57%) were above negligible strength ($rP > 0.2$).

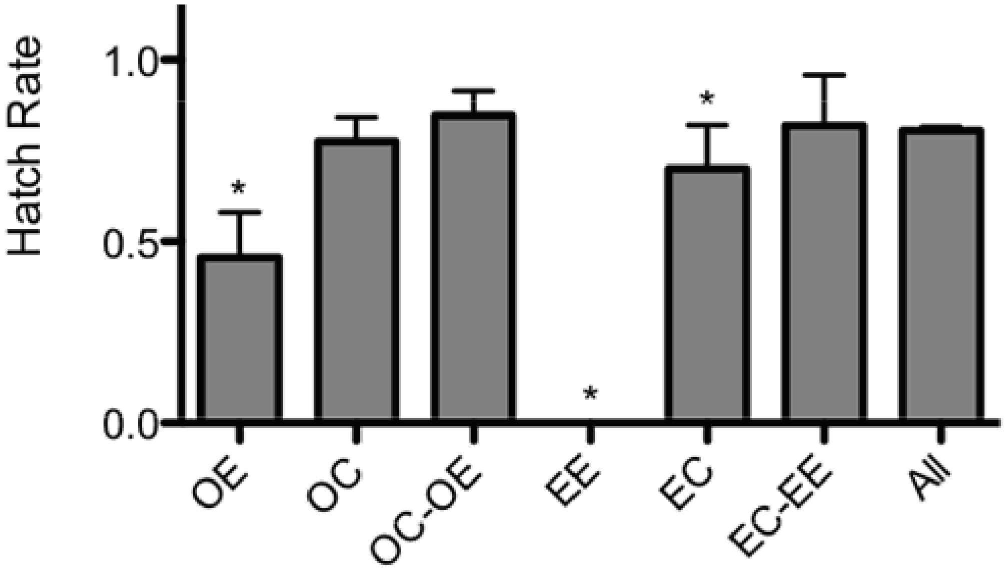

Fig. 2-3. Hatch rates for clutches and eggs in which laying anomalies such as early laying or exclusion of eggs from the coils of the female compared to the hatch rate for all clutches. Specifically, hatch rates for eggs that were found outside the female's coils (OE), clutches that had eggs pushed outside the coils (OC), eggs that were inside the coils from clutches with some eggs laid outside the coils (OC - OE), eggs that were laid early (EE), clutches with eggs that were laid early (EC), eggs that were laid with the majority of the clutch from clutches where some eggs were laid early (EC - EE), and all clutches in this study (All) are analyzed. Asterisks denote hatch rates that are significantly different from the overall hatch rate for all clutches from this study (Mann-Whitney test). Bars above columns represent the standard errors of the means.

Discussion

Information regarding reproductive traits of captive snakes is sparse. Published reports on pythons are limited to small sample sizes, and to discussing averages and ranges for reproductive traits. Previous studies specifically on ball pythons have reported average clutch sizes, duration from ovulation to post-ovulation shed, duration from post-ovulation shed to oviposition, RCM, egg length, egg width, egg mass, and duration of incubation (Barker and Barker, 2006; de Vosjoli et al., 1994; Ross and Marzec, 1990; Van Mierop and Bessette, 1981). These data have been widely used by private and professional python breeders in order to increase breeding efficiencies. Similar data presented in this study (Table 2-1) provide larger sample sizes for these traits, and the results are similar to those published previously (Barker and Barker, 2006; de Vosjoli et al., 1994; Ellis and Chappell, 1987; Ross and Marzec, 1990). In addition, this study provides data on the time duration from last copulation to oviposition.

Table 2-4
Pearson correlation matrix of reproductive traits. Traits include number of healthy offspring per clutch (Healthy Offspring), post-oviposition mass of each female (Female Mass), age of the female at time of oviposition (Age), number of eggs per clutch (Clutch Size), mass of each clutch (Clutch Mass), relative clutch mass (RCM), egg length (EL), and egg width (EW). Strengths of correlations are termed as follows: 0.0 to 0.2, negligible; 0.2 to 0.4, weak; 0.4 to 0.7, moderate; 0.7 to 0.9, strong.

	MAS	Age	CSIZ	CMAS	RCM	EL	EW
HOFF	**0.18**	-0.01	**0.53**	**0.72**	**0.69**	**0.11**	**0.60**
MAS		**0.38**	**0.56**	**0.50**	**-0.14**	**-0.09**	**0.27**
AGE			**0.13**	**0.12**	**-0.12**	0.05	0.05
CSIZ				**0.73**	**0.50**	**-0.46**	**0.27**
CMAS					**0.76**	**0.17**	**0.81**
RCM						**0.17**	**0.67**
EL							**0.48**

Correlations in bold were significant at the $P < 0.05$ level.

For the 783 clutches studied from 2002 to 2009, an average of 97.88 clutches were laid per year. Although ball pythons in this study appeared to generally be pulse breeders, clutches were laid during all weeks of the year except the first 3 weeks in January (Table 2-1). Further, preliminary data suggest that the week of the year a ball python female lays her eggs in captivity is heritable and is significantly affected by both maternal and permanent environmental effects (unpublished data). Reproduction throughout the majority of the year in captive ball pythons is in stark contrast to what has been reported to occur in nature. Wild ball pythons in southern Togo, Africa generally lay their eggs during one month of the year (Aubret et al., 2003). The fact that female ball pythons can proceed through their reproductive cycles at almost any time during the year in captivity could have important implications for those trying to reproduce other python and snake species. Methods such as follicle palpation and ultrasound may significantly enhance success in reproducing these species in captivity by helping to identify times during which males should be introduced to females for copulation (Fig. 2-4 and 2-5). This would be especially important in situations in which keepers are attempting to breed multiple females with single males.

The age at first reproduction (age when oviposition of first clutch occurs) for males in this study varied from 1 to 6 years (average 2.25 years), and for females it varied from 2 to 8 years (average 3.96 years) (Table 2-1). The average reproductive frequency for females was 1.97 years. Although no data has been published on captive or wild ball pythons for these traits, reproductive frequency has been studied and discussed for other python species. Captive reticulated pythons (*Python reticulatus*) and diamond pythons (*Morelia spilota spilota*) have been shown to reproduce every other year (Fitch, 1970; Harlow and Grigg, 1984). Slip and Shine (1988) provided evidence that the reproductive frequency of wild diamond pythons was also likely to be every other year, or potentially even longer. In wild water pythons (*Liasis fuscus*) reproductive frequency is closer to being yearly (Madsen and Shine, 1996). Therefore, a reproductive frequency of every other year in captive ball pythons is similar to that found in other python species.

Aubret et al. (2005) incubated ball python clutches from wild-bred females by three different methods: maternal brooding until hatching (N = 10), maternal brooding for the first 15 days of incubation followed by artificial incubation (N = 10), and artificial incubation only (N = 10). They observed that the more time clutches were artificially incubated, the more desiccated they became, and hatching success decreased. They concluded that artificial incubation led to desiccation and decreased hatching success. During the current study, 27 clutches were not found until they had been laid for 24 hrs or more. When the eggs were found, the dam was brooding them. They were then removed from the females and artificially incubated for the remainder of the incubation period. Statistical analysis on averages for clutch mass, RCM, healthy offspring, hatch rate, egg length, egg width, and hatchling mass between these 27 clutches and all the clutches from this study showed evidence for desiccation and decreased hatching success in the clutches that were found late (Table 2-2). All the traits measured were statistically lower ($P < 0.05$) in the clutches that were found late, except egg length and hatchling mass. Data that suggested desiccation had occurred in clutches that were found late include decreased clutch mass, decreased RCM, and decreased egg width. The decreased hatch rate and number of healthy offspring per clutch suggest lower hatching success in these clutches. In assessment of Aubret et al. (2005), Barker and Barker (2006) suggested that desiccation itself, independent of incubation type, is the cause of decreased hatching success. Because clutches that were found late in this study were desiccated and suffered decreased hatching success even though they were artificially incubated for the majority of the incubation period, these data support the assessment of Barker and Barker (2006).

Fig. 2-4. Ultrasound is being used to determine the stage of follicular growth in this female ball python.

A previous study by Aubret et al. (2003) assessed optimal clutch size in ball pythons. In their study, wild-bred gravid females were caught and brought to a holding facility. Then, ten unmanipulated clutches, nine artificially enlarged clutches (added eggs to increase initial clutch size by 50%), and nine artificially reduced clutches (removed eggs to decrease initial clutch size by 42%) were set up for maternal incubation. Hatching success and hatchling fitness were assessed for the clutches in these three groups. For the clutches that were artificially decreased in size, no benefit to the dam or offspring was detected. However, artificially increasing clutch sizes did significantly decrease hatching success. Therefore, the data from this study suggest that a female’s ability to cover her entire clutch is important to hatching success. During the current study, clutch sizes were reduced by the dam when one or more eggs were laid early, or one or more eggs were excluded from the dam’s coils during brooding. Although the female mass average from females that produced these reduced clutches was not significantly different from the overall female mass average from all clutches laid in this study, clutch mass was significantly higher in clutches that were laid early, and RCM was significantly higher in both types of reduced clutches (Table 2-3). Therefore, clutches were reduced in size when they were large in comparison to female mass (higher RCM), which would potentially lead to females experiencing difficulty in covering the proportionately larger clutches. Also, the hatch rate for clutches that had eggs laid early was significantly lower than the hatch rate for all clutches in this study, but the hatch rate for these same clutches once they were reduced (i.e. not including eggs that were laid early) was not significantly different than the overall average (Fig. 2-3).

Many conclusions can be drawn from the correlations presented in Table 2-4, but a few we find particularly interesting. Age was correlated at the level of rP > 0.2 only with female mass; while female mass was also correlated at rP > 0.2 with clutch size, clutch mass, and EW. This suggests that the mass of the female is more important than age for predicting reproductive output. Further, because female mass was correlated at rP > 0.2 with clutch size and clutch mass, but not RCM, it seems that the proportion of energy allocated to a clutch is independent of the mass of the female even though both the mass of the clutch and the number of eggs in the clutch are moderately correlated with the mass of the female (0.50 and 0.56, respectively). Also of interest, EW was correlated at rP > 0.2 with all traits in the matrix except age. Therefore, EW could be a useful predictor of reproductive output. Lastly, the moderate negative correlation between EL and clutch size, and the weak positive correlation between EW and clutch size support previous research suggesting that as clutch sizes get larger, the eggs get smaller and more round in shape (Brown and Shine, 2007b; Ford and Seigel, 1989; Madsen and Shine, 1996).

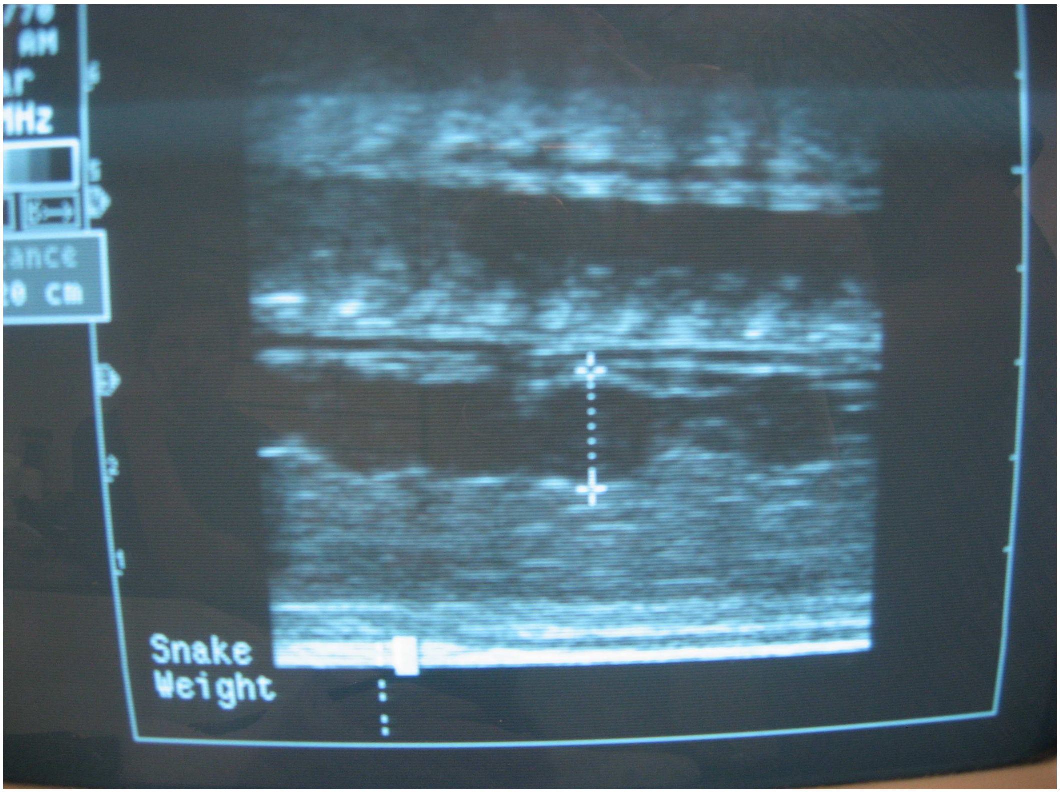

Fig. 2-5. Ultrasound screen image used to count and measure follicles prior to ovulation.

The large sample sizes attained in this study have allowed us to study certain aspects of ball python captive reproduction. Novel data presented in this study provide a foundation for the design of future studies, and for the development of more efficient breeding plans for propagating captive pythons. In addition, some specific information presented here can be of immediate use for python propagation. Results from this study suggest that female ball pythons in captivity ovulate in all months of the year. Also, during this study the female reproductive frequency was every other year. Our results also show that desiccation, even for periods of time as short as only a few days, at the beginning of incubation may significantly decrease hatching success. Taking these findings into account, those attempting to propagate pythons in captivity should do the following: 1) Become proficient in techniques such as follicle palpation and ultrasound in order to assess the reproductive stages of females throughout the year; 2) Understand that the reproductive frequency of python females in captivity may be every other year, or even every third year and not push females to reproduce every year; 3) Make provisions in breeding procedures to decrease the risk of subjecting eggs to desiccation at any time during incubation. With further study of some of the correlations presented in this study (e.g. EW correlations), additional information regarding selection parameters to increase breeding efficiencies may be derived as well. Such knowledge will likely lead to increased success in breeding endangered and otherwise rare and difficult to breed python species in captivity.

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CHAPTER 3

QUANTITATIVE GENETIC ANALYSIS OF BALL PYTHON (*PYTHON REGIUS*) REPRODUCTION TRAITS

Abstract

Ball python reproduction traits (clutch size, clutch mass, relative clutch mass, egg mass, hatch rate, egg length, egg width, hatchling mass, healthy offspring per clutch, week laid, and days of incubation) were recorded for 6480 eggs laid in 937 clutches from 2002 to 2010. For the 937 clutches, the identity of the dam and sire were known for 862 (92%) and 777 (83%) of the clutches, respectively. Univariate analysis allowed for the calculation of repeatability and heritability for these traits. Also, with the use of univariate models we tested whether the inclusion of infertile egg masses when calculating the average egg length and width per clutch was beneficial. Following the construction of the univariate models, a multivariate model that included nine of the eleven traits listed above was compiled. Heritability and genetic and phenotypic correlations were calculated from the multivariate analysis. The statistical significance of various fixed and random explanatory factors were tested in both the univariate and multivariate analyses. The data comparing the use of length and width measurements from all eggs versus all eggs minus infertile egg masses suggested that the use of measurements from infertile egg masses decreased the correlation between these traits and their statistically significant explanatory factors, and yielded lower heritability scores for these traits. The trait that showed the most promise for use in artificial selection to increase reproduction rates was clutch size due to considerable genetic variation, high

heritability, and favorable genetic correlations with other reproduction traits.

Introduction

The captive-bred reptile industry has been growing consistently around the world since the 1970’s (Barker and Barker, 2006; Brant, 2001; Hoover, 1998; Mattioli et al., 2006; Murphy and McCloud, 2010). As an example of the size of the industry, one facility in Florida, USA reported the production of 76,100 captive-bred reptiles, and 2,000,000 rodents marketed for the feeding of captive reptiles, in the year 2001 alone (Brant, 2001). A recent independent economic assessment by Georgetown Economic Services on the captive bred reptile industry in the United States reported that revenues in 2009 were between $1.0 billon and $1.4 billion (Andrew Wyatt, personal communication). Further, Georgetown Economic Services estimated that in 2009 13.6 million reptiles resided in 4.7 million U.S. households. For ball pythons in particular, tens of thousands are produced yearly in captivity, and they are the most commonly kept python species (Barker and Barker, 2006). Also, ball pythons with certain color and pattern mutations have been sold for upwards of $175,000 USD for a single animal (Murphy and McCloud, 2010).

Although the captive reproduction of reptiles has increased significantly over the last few decades, little research has focused on reproduction traits in any captive non-avian reptiles. Among the diminutive body of such research is a series of papers from data collected at the Janamba Croc Farm (Northern Territory, Australia) on saltwater crocodiles (*Crocodylus porosus*) in which they studied reproduction traits (Isberg et al., 2005a), age at slaughter (Isberg et al., 2005b), juvenile survival (Isberg et al., 2006a), and number of scale rows (Isberg et al., 2006b) in relation to skin production. Although sample numbers were sizeable for the reproduction traits studied (30 pairs of breeders and 190 clutches), the researchers were unable to calculate heritabilities (h^2) because they did not know the pedigrees of their breeding adults (all but one pair were wild caught).

A select few smaller studies on wild snake populations and small captive populations have been able to calculate repeatabilities (R) and phenotypic correlations (rP), and in fewer still, genetic correlations (rG) and h^2 (Bronikowski and Arnold, 1999; Brown and Shine, 2007; Farrell et al., 2009; Ford and Seigel, 2006). Unfortunately, these studies generally relied on smaller sample sizes and were plagued with higher error values.

We recently reported data on ball python reproduction traits from a commercial captive breeding facility, The Snake Keeper, Inc. (Utah, USA), that included means, ranges, standard errors, and rP values calculated using least squares (see Chapter 2). Due to the substantial violation of assumptions for the use of least squares to calculate h^2 on this captive population, primary of which was that of random breeding (Akesson et al., 2008; Falconer and Mackay, 1996), we were unable to report h^2 in this initial study.

In the current study we were able to add reproduction data from 2010 and utilize restricted maximum likelihood (REML) to calculate R, h^2, rG, and rP on reproduction traits. The data used in the REML analyses came from 6,480 eggs laid in 937 clutches from 2002 to 2010. For the 937 clutches, the identity of the dam and sire were known for 862 (92%) and 777 (83%) of the clutches respectively. To our knowledge, this is the first report on reproduction traits using REML on any non-archosaurian (crocodilians and birds) reptile species, and the first calculation of h^2 using REML for reproduction traits for any non-avian reptile species.

To date, it has been common practice to include measurements for all eggs in snake clutches when calculating R, h^2, rG, and rP even though it has been documented that snake clutches often contain some infertile egg masses, frequently referred to as “slugs,” that are discolored and smaller than the other eggs (Barker and Barker, 2006; Gorzula et al., 1997; Madsen and Shine, 1996; Ross and Marzec, 1990). We hypothesized that because egg width and length could provide some indication of the female’s oviductal space available for reproduction (Ford and Seigel, 1989; Pizzatto et al., 2007), that the inclusion of infertile egg mass measurements would decrease the correlations between these observations and their explanatory factors, and perhaps decrease heritability for these traits. Therefore, using univariate models we calculated rP estimates between average egg length and width including infertile egg masses (EL+ and EW+) and average egg length and width excluding infertile egg masses (EL and EW) and their statistically significant explanatory factors. We also calculated heritability for EL+, EL, EW+, and EW.

Materials and Methods

Feeding and Environment

Breeder ball pythons were housed within rack systems (Fig. 3-1 and 3-2) in individual cages measuring 81 cm L x 43 cm W x 18 cm H with mesh tops. Chipped aspen bedding was used as the substrate in the cages (Fig 3-3). Whole prey was offered each week and water was available *ad libitum*. From March to October, the rodents offered were approximately 95 g, and during the breeding season (November to February) they were approximately 65 g. The ambient temperature of the breeding facility was controlled to prevent it from exceeding 29.5°C from March to October, and from dropping below 21°C from November to February. Throughout the year, a temperature gradient was maintained in each cage by providing a hot spot that was 32°C during the day and 29.5°C at night. Humidity was constrained in the breeding facility to approximately 60% year round by a Humidifirst MP15 ultrasonic humidifier (Humidifirst, Inc., Boynton Beach, Florida, USA) (Fig. 3-4).

Fig. 3-1. View of the rack systems used for caging in the python production facility. Up to 800 adults and 1000 hatchlings are housed throughout the year in this facility.

Fig. 3-2. Another view of the rack systems in the python production facility.

Breeding and Reproduction

During the breeding season, females over 1500 g were placed in the cages of males over 500 g for one to two days and any observed breeding activity was recorded. An attempt was made to ensure that each female was bred at least once each month during this time. Once females were gravid, they were no longer placed with males.

Females known to be gravid were checked daily for eggs once they were 30 days past their post-ovulation shed. Eggs were removed immediately from each female, weighed as a clutch, counted, separated, weighed individually, measured (length and width), notated if they were infertile egg masses, and placed into an incubation container that was then placed in the incubation room. Each female was also weighed at this time, and a relative clutch mass (RCM) was calculated by dividing the mass of the clutch by the post-oviposition mass of the female. The ages of the sire and dam at the time of oviposition, when known, were also recorded. Also from these data, number of consecutive clutches laid by the female prior to, and including, each clutch was recorded.

Fig. 3-3. Ball python in tub of rack system. Photo by Dan Sutherland.

Incubation and Hatching

The containers used for incubation were Styrofoam shipping containers that measured 28.5 cm L x 39 cm W x 18 cm H externally and were 2.4 cm thick. The medium used for incubation in these containers was a mixture of one part perlite and two parts vermiculite. Then, five parts incubation medium was mixed with one part water by volume and the container was placed in the incubation room (Fig. 3-5) several days prior to incubating eggs in order to allow the contents of the box to reach incubation temperatures. The top of each incubation box was covered with a 1 cm thick pane of glass. The incubation room was temperature controlled by a Helix DBS 1000 (Helix Control Systems, Inc, Vista, California) to stay between 31.4°C and 31.7°C from 2002–2005, and between 30.9°C and 31.1°C from 2006–2010.

Fig. 3-4. A Humidifirst MP15 ultrasonic humidifier is used to maintain the humidity at levels needed for efficient production.

For the eggs that hatched, the hatch date was recorded for each egg and each hatchling was weighed. After hatching, a hatch rate was calculated for each clutch. Also, the number of hatchlings that hatched and did not suffer from any physical abnormalities, such as spinal kinking or eye malformations, was recorded as healthy hatchlings per clutch. For the calculation of average oviposition dates over the years, oviposition dates were reported as number of weeks of the year.

Fig. 3-5. A view of the incubation room at the python production facility. On average, 700 eggs are incubated in this room each year.

Statistical Methods

The identity of the dam and sire were known for 862 (92%) and 777 (83%) of the clutches, respectively, for the 937 clutches recorded in this study. Univariate and multivariate analyses were carried out on the data using ASReml 3.0 (Gilmour et al., 2009). Explanatory factors were removed from univariate and multivariate models by backward elimination when $p > 0.05$. Significance levels for random effects were calculated by running the model with and without the factor and then multiplying the absolute difference between the resulting log-likelihoods by two. This test statistic was assumed to follow a Chi square distribution with one degree of freedom (Gilmour et al., 2009; Pinheiro and Bates, 2000). Repeatability, h^2, and their associated standard errors were calculated first using the univariate models for each trait. Then, a multivariate model was constructed that included nine of the eleven traits. ASReml 3.0 allows for the removal of nonsignificant explanatory factors independently for all traits in the multivariate model. The resulting multivariate model was then used to calculate h^2, rG, and rP for the nine traits in the model. For clutch mass (CMAS) and individual post-oviposition mass (MAS), the data were log-transformed in all statistical analyses.

Univariate Modeling

Table 3-1 contains names, abbreviations, and descriptions for the traits and factors below. The initial univariate model used to evaluate all eleven traits was the following

$$Y_{ijklm} = \mu + YR_k + BA_i + AGE_{ik} + MAS_{ik} + CCL_{ik} + FAC_{ik} + IT_{ijk} + PE_i + MAT_{il} + YBN_i + \varepsilon_{ijklm},$$

where Y_{ijklm} is an observation on CMAS, CSIZ, EL, EW, EL+, EW+, EMASS, HMAS, HOFF, HR, WKLD, INCD, or RCM; μ is the overall mean; YR_k is the fixed effect of the kth year; BA_i is the fixed effect of beneficial alleles of the ith individual; AGE_{ik} is the fixed effect of the age of the ith individual in the kth year; MAS_{ik} is the fixed effect of the mass of the ith individual in the kth year; CCL_{ik} is the fixed effect of consecutive clutches for the ith individual in the kth year; FAC_{ik} is the fixed effect of the facility location of the ith individual in the kth year; IT_{ijk} is the fixed effect of incubation temperature on the clutch from the ith individual and jth sire in the kth year; PE_{ijk} is the random effect of permanent environment on the ith individual; MAT_{il} is the random effect of the lth dam of the ith individual; YBN_i is the random effect of the year the ith individual was born; and ε_{ijklm} is the random residual effect.

Infertile Egg Masses

Correlations were calculated between EL+, EL, EW+, and EW and their statistically significant explanatory factors, except YR because we failed to see any biological reason to test whether the correlations between these traits and YR were affected by inclusion of infertile egg mass measurements. GraphPad Prism 5.0 was used to calculate these correlations. Further, h^2 was calculated for each of these traits using their univariate models in ASReml 3.0.

Multivariate Modeling

In order to construct a multivariate model that would converge, WKLD and INCDYS were dropped out of the model. Explanatory factors that were not significant for each individual trait were removed from the multivariate model as described above. Table 3-2 displays the explanatory factors that were used in the univariate and multivariate models.

Genetic Parameter Estimates

Repeatability, h^2, rG, and rP were calculated as described in Wilson et al. (2009). Briefly, variance components calculated in ASReml 3.0 were used as follows

Repeatability

$R = V_I/V_P$

where V_I is the individual variance and V_P is the phenotypic variance;

Heritability

$h^2 = V_A/V_P$

where V_A is the additive genetic variance and V_P is the phenotypic variance;

Genetic correlation

$rG = V_{A1,A2}/\sqrt{V_{A1} * V_{A2}}$

where $V_{A1,A2}$ is the additive genetic covariance of traits 1 and 2, V_{A1} is the additive genetic variance of trait 1, and V_{A2} is the additive genetic variance of trait 2.

Phenotypic correlation

$rP = V_{P1,P2}/\sqrt{V_{P1} * V_{P2}}$

where $V_{P1,P2}$ is the phenotypic covariance of traits 1 and 2, V_{P1} is the phenotypic variance of trait 1, and V_{P2} is the phenotypic variance of trait 2;

Results

Univariate Analyses

The univariate models that included the fixed and random explanatory factors displayed in Table 2-2 were used to estimate R, h^2, and their associated standard errors (Table 3-3). The factor YR was a significant addition to the univariate models for all traits except WKLD. Further, the univariate model for WKLD was the only model that had any random effects fitted to it. The following factors were only fitted to one of the models: CCL was fitted to the CSIZ model, FAC was fitted to the EW model, and IT was fitted to the INCD model. The estimates for R and h^2 for each trait, except WKLD, were nearly identical.

Table 3-1
Names, abbreviations, and descriptions for traits and fixed and random explanatory factors used in this study.

Names	Abbreviations	Descriptions
Traits		
Week Laid	WKLD	Week of the year the clutch was laid
Hatchling Mass	HMAS	Mean hatchling mass per clutch
Egg Mass	EMAS	Mean egg mass per clutch
Egg Length	EL	Mean egg length per clutch
Egg Width	EW	Mean egg width per clutch
Egg Length Plus	EL+	Mean egg length per clutch, including infertile masses
Egg Width Plus	EW+	Mean egg width per clutch, including infertile masses
Relative Clutch Mass	RCM	Mass of the clutch divided by the post-oviposition mass of the female
Clutch Mass	CMAS	Mass of the clutch
Clutch Size	CSIZ	Number of eggs (fertile and infertile) per clutch
Healthy Offspring	HOFF	Number of healthy offspring per clutch
Hatch Rate	HR	Number of offspring hatched per clutch
Incubation Days	INCD	Number of days of incubation per clutch
Fixed Factors		
Year	YR	Year clutch was laid
Dam Age	AGE	Age of dam when clutch was laid
Dam Mass	MAS	Post-ovulation mass of dam
Consecutive Clutches	CCL	Number of consecutive years female has laid eggs
Beneficial Alleles	BA	Number of color and pattern alleles that increase the value of the female
Facility	FAC	Facility where reproduction took place
Incubation Temperature	IT	Temperature at which clutch was incubated
Random Factors		
Permanent Environment	PE	Permanent environmental effects on the dam
Maternal Effect	MAT	Maternal effect of the maternal grand dam on the dam
Year Born	YBN	Year the female was born

Table 3-2
Fixed and random explanatory factors that were significant additions to the univariate and multivariate models. Fixed effects were chosen by backwards elimination when $p > 0.05$, and random effects were chosen using the likelihood test ratio explained above. Levels of significance are as follows: $0.01 < p < 0.05$ (*); $0.001 < p < 0.01$ (**); $p < 0.001$ (***); and a dash (-) denotes factors that were not significant. A superscript M denotes traits that remained significant in the multivariate model. Table 3-1 displays the abbreviations and descriptions for the traits and effects.

	Fixed Terms							Random Terms		
Trait	YR	BA	AGE	MAS	CCL	FAC	IT	PE	MAT	YBN
CMAS	***^{M}	**^{M}	-	***^{M}	-	-	-	-	-	-
CSIZ	***^{M}	*	-	***^{M}	*	-	-	-	-	-
EL	***^{M}	-	***^{M}	-	-	-	-	-	-	-
EMAS	***^{M}	-	**	***^{M}	-	-	-	-	-	-
EW	***^{M}	-	-	***^{M}	-	***^{M}	-	-	-	-
HMAS	***^{M}	-	*	***^{M}	-	-	-	-	-	-
HOFF	***^{M}	***^{M}	-	-	-	-	-	-	-	-
HR	***^{M}	-	-	-	-	-	-	-	-	-
RCM	***^{M}	*	-	**	-	-	-	-	-	-
INCD	***	***	-	-	-	-	***	-	-	-
WKLD	-	*	*	-	-	-	-	*	*	-

The rP between EL and AGE was higher than the rP for EL+ and AGE (0.13 and 0.08, respectively; Table 3-4). Similarly, the rP between EW and MAS was higher than the rP for EW+ and MAS (0.53 and 0.35, respectively; Table 3-4). The rP estimates between FAC and both EW and EW+ were not significant ($p > 0.2$). Lastly, the h^2

estimates for egg length and width were higher when infertile egg masses were excluded from the calculation of clutch averages for these traits (0.45 versus 0.35; Table 3-4).

Table 3-3
Repeatability, heritability, and their associated standard errors calculated using univariate models.

Trait	R	SE_R	h^2	SEh^2
CMAS	0.25	0.05	0.25	0.05
CSIZ	0.43	0.05	0.43	0.05
EL	0.48	0.05	0.48	0.05
EMAS	0.57	0.05	0.57	0.05
EW	0.45	0.05	0.45	0.05
HMAS	0.45	0.06	0.45	0.05
HOFF	0.29	0.05	0.29	0.05
HR	0.31	0.05	0.31	0.05
RCM	0.31	0.05	0.31	0.05
INCD	0.25	0.06	0.25	0.06
WKLD	0.35	0.10	0.12	0.15

In the multivariate analysis, YR was a significant factor for all nine traits within the model. The number of beneficial alleles that a female had (adjusting for preferential treatment of females of greater worth) was a significant factor only for CMAS and HOFF. Post-oviposition mass of the female was a significant factor for five traits

(CMAS, CSIZ, EMAS, EW, and HMAS), while AGE was only a significant factor for EL (Table 3-3). The factor FAC remained significant for EW.

Table 3-4
Comparison of phenotypic correlations between EL+ and EL, and EW+ and EW and the significant explanatory factors in their respective univariate models, and their heritability estimates. Correlations followed by an asterisk (*) were significant at a $0.01 < P < 0.05$; (***) were significant at a $p < 0.001$; and (NS) were not significant ($P > 0.05$). Factors that were not selected for use in the univariate model of a given trait are denoted by NA. Heritability estimates are followed by their standard errors in parentheses.

Trait	AGE	MAS	FAC	h^2 (SE)
EL+	0.08*	NA	NA	0.47 (0.05)
EL	0.13***	NA	NA	0.48 (0.05)
EW+	NA	0.35***	0.04^{NS}	0.35 (0.05)
EW	NA	0.53***	0.01^{NS}	0.45 (0.05)

The sample size, minimum value, maximum value, mean, standard error of the mean, and coefficient of variation (standard deviation divided by the mean) are provided for each trait from the multivariate analysis in Table 3-5. Table 3-6 displays the h^2, rG, and rP for each of the traits in the multivariate analysis along with their associated standard errors. From the multivariate model, estimates of h^2 ranged from 0.21 to 0.60; positive and negative rG values ranged from negligible (0.002, -0.006) to strong (0.96, -0.71).

Discussion

Significant Explanatory Factors

Year was a significant factor for all traits in both the univariate and multivariate analyses, except for WKLD. The post-ovulation mass of the female was a significant factor in the multivariate model for CMAS, CSIZ, EMAS, EW, and HMAS. Surprisingly, AGE was only a significant factor in the multivariate model for EL. This is especially interesting because the multivariate analysis suggests that in our population MAS is a significant factor for EW, while AGE is a significant factor for EL. This could be due to the fact that we did not adjust MAS for SVL and likely, due to indeterminant growth, AGE and SVL are positively correlated, as has been shown in many reptiles, including several snake species (Baron et al., 2010; Farrell et al., 2009; Ford and Seigel, 1994). Therefore, age would likely have a higher correlation with SVL than mass would. Thus, as a female gets longer with age, the eggs in her reproductive tract may be able to be more elongate. On the other hand, EW was affected more by MAS, which could mean that heavier females were able to allocate more resources to their eggs, and given the allometric constraints described by Ford and Seigel (1989), those eggs likely would become wider and shorter. This is supported by findings in multiple snake species in which increased food intake caused significant increases in EW (Brown and Shine, 2002; Ford and Seigel, 1994; Seigel and Ford, 1991).

Multivariate Analysis

The only trait that the change in facilities from California (CA, USA) to Utah (UT, USA) affected was EW. Best linear unbiased estimators for the factor FAC showed that EW was higher for UT clutches than for CA clutches (unpublished data). Research in wild and captive snakes has shown that females that have higher food intake have significantly wider eggs (Brown and Shine, 2002; Ford and Seigel, 1994; Seigel and

Ford, 1991). Because we did not record food intake and were thus unable to adjust for it, an average increase in food intake for females in UT in comparison to those in CA may be a viable explanation for increased average EW for clutches produced in UT.

Table 3-5
Sample size (N), minimum value (Min), maximum value (Max), mean (Mean), standard error of the mean (SEM), and coefficient of variation (CV) for each of the traits used in the multivariate model. Clutch mass has been log transformed. All other masses are given in grams. Lengths and widths are given in millimeters.

Trait	N	Min	Max	Mean	SEM	CV
CMAS	822	1.96	3.10	2.76	0.01	0.06
CSIZ	830	3	14	6.92	0.06	0.26
EL	722	58	100	77.58	0.22	0.08
EMAS	645	48	135	94.46	0.47	0.13
EW	722	35	54	46.74	0.10	0.06
HMAS	667	27	90	62.59	0.32	0.13
HOFF	749	0	12	5.52	0.09	0.44
HR	749	0	1	0.81	0.01	0.36
RCM	830	0	0.71	0.41	< 0.01	0.26

Female frequency of reproduction is often discussed among ball python breeders. Barker and Barker (2006) suggested that limiting females to breeding every other year may increase overall production. In our univariate model we did detect a significant effect of consecutive clutches (females laying clutches in two or more consecutive years) on clutch size (Table 3-3). Although in the multivariate model this effect was not significant (Table 3-5). We were unable to find any convincing evidence from our data to suggest that breeders should purposely limit their females to breeding every other year.

Table 3-6
Estimates of h^2, rP, and rG and their associated standard errors for the traits used in the multivariate analysis. Estimates of h^2 are bolded and displayed along the diagonal; estimates of rG are given above the diagonal; and estimates of rP are given below the diagonal. Each estimate of h^2, rG and rP is followed by its standard error in parentheses. Table 3-1 provides the names and descriptions for the abbreviations of the traits.

	CSIZ	CMAS	RCM	EMAS	HR	EL	EW	HMAS	HOFF
CSIZ	**0.44 (0.05)**	0.40 (0.11)	0.46 (0.09)	-0.61 (0.07)	-0.25 (0.12)	-0.71 (0.06)	-0.29 (0.11)	-0.61 (0.08)	0.54 (0.09)
CMAS	0.50 (0.03)	**0.21 (0.05)**	0.96 (0.02)	0.30 (0.11)	0.61 (0.11)	0.08 (0.14)	0.55 (0.10)	0.35 (0.11)	0.77 (0.08)
RCM	0.60 (0.03)	0.87 (0.01)	**0.24 (0.05)**	0.27 (0.11)	0.49 (0.11)	-0.01 (0.13)	0.57 (0.09)	0.29 (0.11)	0.70 (0.08)
EMAS	-0.40 (0.04)	0.26 (0.04)	0.24 (0.04)	**0.60 (0.04)**	0.42 (0.09)	0.88 (0.03)	0.81 (0.05)	0.96 (0.01)	-0.13 (0.12)
HR	0.02 (0.04)	0.57 (0.03)	0.57 (0.03)	0.31 (0.04)	**0.28 (0.05)**	0.40 (0.12)	0.43 (0.11)	0.54 (0.09)	0.65 (0.08)
EL	-0.63 (0.03)	-0.10 (0.04)	-0.15 (0.04)	0.79 (0.02)	0.13 (0.04)	**0.46 (0.05)**	0.49 (0.10)	0.79 (0.05)	-0.23 (0.12)
EW	0.14 (0.04)	0.59 (0.03)	0.62 (0.03)	0.63 (0.03)	0.37 (0.04)	0.17 (0.04)	**0.39 (0.05)**	0.86 (0.05)	0.14 (0.13)
HMAS	-0.34 (0.04)	0.34 (0.04)	0.32 (0.04)	0.89 (0.01)	0.42 (0.03)	0.67 (0.02)	0.63 (0.03)	**0.51 (0.04)**	0.00 (0.12)
HOFF	0.54 (0.03)	0.66 (0.02)	0.73 (0.02)	0.01 (0.04)	0.79 (0.02)	-0.23 (0.04)	0.34 (0.04)	0.13 (0.04)	**0.24 (0.05)**

Egg Length and Width and the Inclusion of Infertile Egg Masses

We hypothesized that egg width, and perhaps egg length, could provide an indication of the volume within the oviduct available for eggs, as has been discussed by other researchers (Ford and Seigel, 1989; Pizzatto et al., 2007). Due to the fact that snakes do oviposit some infertile masses among their clutches that are smaller (Barker and Barker, 2006; Gorzula et al., 1997; Madsen and Shine, 1996; Ross and Marzec, 1990), we hypothesized that the inclusion of the measurements for these infertile, smaller egg masses would decrease the correlations between these traits and their explanatory factors because these smaller masses would be poor indicators of oviductal space. Further, we hypothesized that averages for egg width and perhaps egg length that included measurements from infertile egg masses would lead to lower h^2 estimates.

Our data provide evidence that it would likely be beneficial for researchers to exclude measurements from infertile egg masses when they are calculating mean egg lengths and widths for use in developing breeding selection programs for ball pythons (Table 3-4), and perhaps other snake species as well. Heritability for egg width increased from 0.35 to 0.45 (Table 3-6) when infertile egg mass measurements were removed, and the correlation between egg width and its explanatory factor MAS was higher when infertile egg masses were removed as well (0.35 versus 0.53; Table 3-6). Although the increase in h^2 for egg length was less dramatic (0.47 to 0.48; Table 3-6), the correlation with its explanatory factor AGE was also increased (0.08 to 0.13; Table 3-6). Given these results, we used the egg length and width means that did not include measurements from infertile egg masses in the multivariate model. Moreover, we suggest that other researchers working with ball pythons, and perhaps other snake species, do the same.

Evaluation of Traits for Use in Selection Criteria

Maximization of HOFF was deemed to be our main goal. Estimates from the multivariate analysis for h^2 ranged from 0.21 to 0.60 (Table 3-6), and for CV ranged from 0.06 to 0.44 (Table 3-5). Although the highest CVs were for HOFF and HR, they were only of moderate heritability (0.24 and 0.28 respectively). While the heritability for EMASS was the highest of all the traits (0.60), CV for EMAS was only 0.13. Further, the rG and rP for EMASS and HOFF were -0.13 and 0.009, respectively. Therefore, although HOFF, HR, and EMAS were deemed important for use in creating selection criteria, they were not ideal due to lower heritability or genetic variation. Conversely, heritability for CSIZ was high (0.44), and the estimate of CV for CSIZ was among the highest for all the traits (0.26). Also, rG and rP between CLSIZE and HOFF were both 0.54. Given the above data, CSIZ appeared to be the most ideal trait to focus on when setting up selection criteria for this captive population of ball pythons.

A trade-off between clutch size and egg size has been shown in many species, including several snake species (Bonnet et al., 2001; Brown and Shine, 2007; Ford and Seigel, 2006; Garner et al., 2002; Gregory and Skebo, 1998; King, 1993; Li-xin et al., 2006). For captive ball pythons this trade-off seems to exist as well. The rG estimates between CSIZ and EMAS, EL, and EW were -0.61, -0.71, and -0.29, respectively. The rP estimates between CSIZ and EMAS, EL, and EW were -0.40, -0.63, and 0.14, respectively. Therefore, if selection pressure is applied to produce larger clutch sizes, breeders should pay attention to potential decreases in EMAS because increasingly smaller egg masses will likely decrease hatch rates (rG = 0.42 between EMAS and HR) and potentially lead to decreased HOFF (rG = 0.65 between HOFF and HR).

Comparison of reproduction data from wild populations of ball pythons and our captive population suggest that improvements in clutch size are feasible (Table 3-7). Data from two different research groups on wild ball python reproduction traits report values for average clutch size that are significantly higher than that found in our captive population (Aubret et al., 2003, 2005c; Gorzula et al., 1997). Further, both research groups report estimates for RCM in the wild populations they studied that were significantly higher than that found in our captive population. The females in our study were, on average, higher in mass but produced clutches of lower mass than those in either of the two wild populations. Therefore, improvement in clutch size and female reproductive efficiency should be achievable through artificial selection.

Conclusions

Clutch size was identified as the best candidate for use in selection programs for the study population of ball pythons. Other traits that deserve attention in developing selection criteria include hatch rate, healthy offspring per clutch, and egg mass. Our data provide evidence that researchers should exclude measurements from infertile egg masses when calculating mean egg lengths and widths for use in ball python selection programs.

Table 3-7

Comparison of reproduction data between our captive population and data from studies on wild populations. All values are means per clutch followed by their respective standard deviations in parentheses. Student's t-tests were used to compare means between the two wild populations and our captive population. Means from wild populations that were significantly different from the means calculated from our captive population at a level of $0.01 < p < 0.05$ are denoted by an asterisk (*), or three asterisks (***) when $p < 0.001$. All values from Aubret et al. came from their 2003 paper except EMAS which came from Aubret et al. 2005. All masses are given in grams. Lengths and widths are given in millimeters.

Traits	Current Study	Gorzula et al., 1997	Aubret et al., 2003, 2005
CSIZ	6.9 (1.8)	8.1 (1.7)***	7.7 (1.7)***
MAS	1487 (322)	1337 (238)*	1235 (241)***
CMAS	609 (196)	772 (138)***	646 (174)*
RCM	0.41 (0.11)	0.55 (0.07)***	0.52 (0.09)***
EMAS	94.5 (11.8)	97.9 (16.3)	90.0 (10.7)*

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CHAPTER 4

TWINNING IN REPTILES: EVIDENCE OF RELATIVELY HIGH RATES OF MONOZYGOTIC TWINNING AND SURVIVAL OF TWINS IN SNAKE SPECIES

Abstract

Although large datasets have been published for twinning in avian species, relatively few are available for non-avian reptiles. Such reports, to date, have been restricted mostly to chelonian species. From the chelonian and avian data it has been generally concluded that twinning is reproductively disadvantageous because of high mortality rates experienced by twins (usually over 50%). Also, conjoined twinning rates in chelonian and avian species are generally higher than rates for complete twinning, and some reports mention the size of the twin-bearing egg being larger than the other eggs in the clutch. A paucity of research has focused on the differences in reproductive traits between females that produce twinning and non-twinning clutches, and no reports have been published that provide genetic evidence of monozygotic twinning in any non-avian reptile species. We report that 14 sets of twins were produced from 6,480 eggs from 937 ball python (*Python regius*) clutches. The survival rate for twins during the first 3 months of life in our study was 97%. Further, we did not observe any instances of conjoined twinning in the 6,480 ball python eggs studied, nor did we detect any difference between the sizes of twin- and non-twin-bearing eggs. We also tested for differences in reproductive traits between twinning and non-twinning clutches (age of female, clutch size,

female mass, clutch mass, relative clutch mass, and incubation period) and found that relative clutch mass for twinning clutches was significantly higher than for non-twinning clutches. Interestingly, 11 of the sets of twins were identical in sex and phenotype, and we present additional genetic data that further suggests the rate of monozygotic twinning within our captive population of ball pythons was higher than that of dizygotic twinning. Further, using microsatellite analysis we were able to generate data that shows three sets of python twins were genetically identical.

Introduction

Large datasets are available on twinning rates for avian species (Byerly and Olsen, 1934; Sittmann et al., 1971). Extensive datasets on twinning in non-avian reptile species are mostly limited to chelonian species (Eckert, 1990; Hildebrand, 1938; Tucker and Janzen, 1997; Yntema, 1970, 1971). Reports of twinning in the remaining non-avian reptile groups consist mainly of accounts of single occurrences of twinning (Aucone and Branham, 2005; Blomberg, 1979; Carpenter and Yoshida, 1967; Clark and Tytle, 1983; Curtis, 1950; Gudynas and Gambarotta, 1981; Hartdegen and Bayless, 1999; Mackness et al., 1998; Marion, 1980; Reese, 1906; Shaw, 1954; Shuette, 1978).

From the large turtle and bird datasets, reports have shown twinning to be reproductively disadvantageous due to low survival rates experienced by the twins. In turtles, a major contributor to the low survival rates observed was that a high percentage, estimated to be approximately 80%, of the twins found were asymmetrical and the smaller twin died in the egg (Tucker and Janzen, 1997;

Yntema, 1970, 1971). Studies consisting collectively of 4,943 red-eared slider (*Trachemys scripta*) and over 6,000 common snapping turtle (*Chelydra serpentina*) eggs reported survival rates of twins to be between 40% and 50% (Tucker and Janzen, 1997; Yntema, 1970, 1971). Hildebrand (1938) reported finding only one set of completely separate twins from 100,000 diamond-back terrapin (*Malaclemmys centrata*) eggs, and they both died shortly after being found. From a study that consisted of approximately 40,000 leatherback sea turtle (*Dermochelys coriacea*) eggs it was concluded that all twins perished before hatching (Eckert, 1990). Likewise, studies on multiple avian species reported 0% survival rates for twinned embryos (Munro, 1965; Sittmann et al., 1971).

A higher occurrence of conjoined twinning in comparison to complete twinning has been reported in the turtle and bird literature (Byerly and Olsen, 1934; Crooks and Smith, 1958; Hildebrand, 1938; Sittmann et al., 1971; Yntema, 1970, 1971). Also, reports of twin eggs being larger than the other eggs from the same clutches have come from several reptilian taxonomic groups including the following: avian (Alley and Berry, 2002; Bassett et al., 1999); crocodilian (Blomberg, 1979); lizard (Carpenter and Yoshida, 1967; Hartdegen and Bayless, 1999); and colubrid snake (Singh and Thapliyal, 1973).

In two different turtle studies, attempts were made to investigate for potential differences between twinning and non-twinning clutches and females (Eckert, 1990; Tucker and Janzen, 1997). Eckert (1990) found no differences in female size, clutch size, incubation period, or year associated with twin-bearing clutches compared to those without twins. Conversely, she did find that twin-bearing clutches did have a significantly higher percent of yolked eggs than non-twinning clutches, and females that produced two or more twins in a single year were 17 times more likely to twin again the following year than by chance alone. Tucker and Janzen (1997) reported that twinning females were larger in plastron length and mass, and laid larger clutches than non-twinning females. We were unable to find any similar studies on any other non-avian reptile taxonomic groups.

In mammals the rate of monozygotic twinning is generally lower than that for dizygotic twinning (reviewed in Aston et al., 2008; Gleeson, 1994; although see Blickstein and Keith, 2007 for a notable exception). In the turtle literature more researchers have surmised that the twinning they have observed has been dizygotic (Crooks and Smith, 1958; Yntema, 1970, 1971) than those concluding observed twins were monozygotic (Hildebrand, 1938). Conversely, among snake species more researchers have concluded observed twinnings were monozygotic (Curtis, 1950; Mackness et al., 1998; Manimozhi et al., 2006) than dizygotic (Marion, 1980). However, to date no studies have reported genetic data showing monozygotic twinning to have occurred in any non-avian reptile species.

Materials and Methods

Study Animals

Ball pythons were housed by commercial breeders, The Snake Keeper, Inc. (UT, USA). Captive husbandry for the ball pythons was as described previously (see Chapter 2). Briefly, females were checked daily for eggs. Once eggs were found they were removed from the female, counted, and weighed both individually and as a clutch. Individual egg lengths and widths were also recorded. Also, the age and post-oviposition mass of the females were recorded. In all cases, clutches of eggs were artificially incubated individually in insulated containers containing a perlite, vermiculite, and water mixture. The containers were then placed within a temperature controlled incubation room until hatching. At hatching, all instances of twinning were recorded. The mass, sex, and color/pattern of all hatchlings were also recorded. Twins were regarded as asymmetrical if the mass of the smaller twin was less than 80% of the larger twin. In addition, it was noted which eggs the twins came from so initial egg mass, egg length, and egg width were known for twinning versus non-twinning eggs. All eggs that did not hatch were manually pipped and the contents were investigated for additional twins. All live twins were housed, fed, and observed for at least three months before leaving the facility.

Microsatellite Data

Shed skins were collected from the sire, dam, and both twins of one and two sets of ball python and carpet python (*Morelia spilota*) twins respectively. The shed skins were allowed to dry at room temperature and were subsequently placed in Ziplock® bags and stored at room temperature until DNA was extracted. Extraction of DNA was carried out as described in Fetzner (1999), which mostly follows the protocol provided in the Puregene® DNA isolation kit.

Amplicons for fragment length analysis of microsatellites were produced using a one-step nested PCR method (Schuelke, 2000). The microsatellite primers used in this study were specifically developed for use on python samples with this nested PCR method (Jordan et al., 2002). Jordan et al. (2002) report the sequences for the primers utilized in the current study under the same names. Polymerase chain reactions consisted of 12.5 µl Promega GoTaq® Master Mix, 0.7 µl of forward and reverse primer mix (5.6 pmol M13-labeled primer, 8.4 pmol non-labeled primer), 0.5 µl of 6FAM-labeled M13 (8.4 pmol), and 500 ng of template DNA and water to a final volume of 25 µl. The PCR cycling parameters used were as follows: 95°C for 9 min; then 94°C for 45 sec, annealing at 65°C for 45 sec, and 72°C for 1 min was repeated 15 times with a 1°C decrease in annealing temperature for each subsequent cycle; 94°C for 45 sec, 50°C for 45 sec, and 72°C for 1 min was repeated 20 times; 72°C for 10 min (Taylor, 2005). Samples were then submitted directly to the Center for Integrated Biosystems Genomics Core at Utah State University for fragment length analysis. An ABI 3730 DNA Analyzer was used to generate the fragment length analysis data.

Data Analysis

Female mass and clutch mass were log-transformed prior to use in any statistical analyses. Student's t-tests and paired t-tests were analyzed using GraphPad Prism 5.0. The microsatellite data were analyzed using STRand version 2.4.55.

Results

Of the 6,480 ball python eggs examined in this study, 14 contained sets of twins. The eggs came from 14 clutches sired by 14 different males and laid by 13 different females. One female laid two clutches that contained sets of twins, one in

2003 and another in 2007. These were the only two years this female laid eggs during the study. Table 4-1 displays sample size, percent twins per egg, percent twins per clutch, and percent of females that produced twins from our study and other studies on bird and turtle species in which sample sizes were of 800 eggs or more.

Table 4-1
Twinning rates from studies consisting of more than 800 eggs.

Species	Source	Number of eggs	% of eggs with twins	% of clutches with twins	% of females producing twins
Avian					
Gallus gallus	1	122,362	0.002	-	-
	2	1,376	0	-	-
Coturnix coturnix	2	2,403	0.92	-	-
Notiomystis cincta	3	830	0.12	0.5	-
Chelonian					
Dermochelys coriacea	4	40,000	0.03	2.6	10.8
Chelydra serpentina	5	5,074	0.63	19	19
	6	1,289	0.16	6.9	6.9
Trachemys scripta	6	4,943	0.20	2.4	2.4
Malaclemmys centrata	7	100,000	0.001	-	-
Terrapene carolina	8	826	0.12	0.4	0.4
Ophidian					
Python regius	9	6,480	0.22	1.5	2.5

Sources: (1) Byerly and Olsen, 1934; (2) Sittmann et al., 1971; (3) Thorogood and Ewen, 2006; (4) Eckert, 1990; (5) Yntema, 1970; (6) Tucker and Janzen, 1997; (7) Hildebrand, 1938; (8) Messinger and Patton, 1995; (9) current study

Table 4-2 provides reproductive, egg, and hatchling traits associated with the 14 twin-bearing clutches. Paired t-tests were used to test for differences between twinning and non-twinning egg length, egg width, and egg mass. All P-values from these t-tests were insignificant ($P > 0.25$). A similar paired t-test that was estimated to investigate for potential differences between the combined hatchling mass of twins and the average mass of their non-twin siblings also yielded an insignificant P-value ($P > 0.50$).

Student's t-tests yielded insignificant P-values for differences between twinning and non-twinning clutches for age of the female, post-oviposition mass of the female, clutch size, clutch mass, and incubation period ($P > 0.05$) (Table 4-3). Conversely, relative clutch mass (clutch mass divided by post-oviposition mass) for twinning clutches was significantly higher than that for non-twinning clutches ($P = 0.02$).

The sex of both individuals was recorded for 11 sets of twins that were suspected to be monozygotic twins by their identical color/pattern phenotypes that are known to be genetically inherited (Barker and Barker, 2006). In all 11 cases both individuals were of the same sex. The probability of observing 11 sets of same sex pairs under the null hypothesis that none of the 11 sets were monozygotic twins would be 2.4×10^{-7} (Table 4-4). Further, including the knowledge of modes of inheritance for color/pattern morphs and the phenotypes of the adults and twins, the probability of observing 11 sets of twins of the same sex and phenotype as we found if none of the sets of twins were monozygotic would be 3.1×10^{-17} (Table 4-4).

Table 4-2

Reproductive, egg, and hatchling traits for twins and siblings from 14 twinning clutches. Egg and hatchling traits for twins are in bold. TWCL = twin clutch number; YR = year that the clutch was laid; AGE = age of the dam at the time the clutch was laid; MAS = post-oviposition mass of the dam; CSIZ = clutch size (number of eggs in the clutch, including infertile egg masses); CMAS = clutch mass; RCM = relative clutch mass (CMAS divided by MAS); INCD = number of days eggs were incubated; EMAS = mass of fertile eggs; EL = egg length; EW = egg width; HMAS = hatchling mass; SEX = sex of both twins; F = female; M = male. All measurements of mass are in grams, length and width measurements are in millimeters, and dam age is in years. An asterisk (*) denotes sets of twins that were regarded as being asymmetrical. The mass of the second twin from TWCL 13 is missing because this individual died early in development.

TWCL	YR	AGE	MAS	CSIZ	CMAS	RCM	INCD	EMAS	EL	EW	HMAS	SEX
1	2003	7	1353	8	689	0.51	-	-	**71**	**47**	**25/23**	**-**
							-	-	77	46	54	
2	2005	5	1238	7	702	0.57	**59**	**107**	**88**	**46**	**38/32**	**F**
							59	98	78	47	68	
3	2007	7	1384	8	737	0.43	**58**	**92**	**79**	**45**	**33/31**	**F**
							59	91	79	45	61	
4	2007	7	1460	8	759	0.52	**58**	**88**	**74**	**46**	**33/23***	**M**
							58	95	76	49	61	
5	2007	6	1781	10	975	0.55	**59**	**92**	**69**	**52**	**34/29**	**M**
							60	97	74	51	63	
6	2007	6	1485	9	804	0.54	**56**	**-**	**74**	**47**	**41/20***	**M**
							55	-	73	47	63	
7	2007	4	986	6	558	0.57	**58**	**84**	**79**	**45**	**29/25**	**M**
							58	93	79	46	60	
8	2007	4	1384	6	570	0.41	**59**	**98**	**79**	**45**	**35/33**	**M**
							59	93	74	47	63	
9	2007	10	1481	5	498	0.34	**57**	**103**	**91**	**44**	**38/27***	**M**
							57	97	83	45	64	
10	2007	3	1089	6	473	0.43	**55**	**74**	**59**	**46**	**33/31**	**M**
							55	80	70	46	56	
11	2008	4	1795	6	584	0.33	**57**	**93**	**71**	**50**	**28/22***	**M**
							56	97	75	47	65	
12	2010	7	1852	6	805	0.43	**60**	**130**	**96**	**49**	**48/42**	**F**
							59	120	84	54	84	

Table 4-2 (continued)

TWCL	YR	AGE	MAS	CSIZ	CMAS	RCM	INCD	EMAS	EL	EW	HMAS	SEX
13	2010	7	1666	8	846	0.51	**63**	**103**	**80**	**49**	**23/-***	**-**
							64	106	76	52	64	
14	2010	8	1850	9	954	0.52	**61**	**104**	**80**	**52**	**41/19***	**M**
							61	106	78	52	76	

Table 4-3
Comparison of female reproductive traits between twinning and non-twinning clutches. Female age is given in years. Values for female mass and clutch mass are log-transformed. Student's t-test was used to calculate the P-values displayed.

Trait	All Clutches	Twinning Clutches	P-value
Female Age	6.19 (2.53)	6.07 (1.90)	0.86
Female Mass	3.16 (0.09)	3.16 (0.08)	0.93
Clutch Size	6.92 (1.77)	7.29 (1.49)	0.44
Clutch Mass	2.76 (0.16)	2.84 (0.10)	0.05
RCM	0.42 (0.10)	0.48 (0.08)	0.02
Incubation Days	59.09 (1.88)	59.67 (2.24)	0.27

Given the above observations of identical sex and color/pattern morphs in 11 sets of twins we decided to use a molecular assay to see if we could produce the first genetic evidence of monozygotic twinning in a non-avian reptile species. The microsatellite data from one set of ball python twins and two sets of carpet python twins are presented in Table 4-5. Probabilities, under the null hypothesis that each of the individual sets of twins were not monozygotic, that combined microsatellite, color/pattern, and sex data for the set of ball python twins and the two sets of carpet python twins were 9.8×10^{-4}, 6.1×10^{-5}, and 3.4×10^{-5}, respectively (Table 4-5). Therefore, the probability that none of these three sets of twins were monozygotic would be 2.0×10^{-12}.

Discussion

The majority of twinning data published to date for reptilian species come from bird and turtle species. Datasets of tens of thousands to over one hundred thousand eggs are available for these taxa (Byerly and Olsen, 1934; Eckert, 1990; Hildebrand, 1938). Twinning reports from other taxa consist almost exclusively of single twinning events (Aucone and Branham, 2005; Blomberg, 1979; Carpenter and Yoshida, 1967; Clark and Tytle, 1983; Curtis, 1950; Gudynas and Gambarotta, 1981; Hartdegen and Bayless, 1999; Mackness et al., 1998; Marion, 1980; Reese, 1906; Shaw, 1954; Shuette, 1978). We present data from 6,480 eggs from 937 ball python clutches in which 14 cases of twinning were observed.

Reported rates for complete twinning events per egg in bird species range from 0% to 0.12% (Byerly and Olsen, 1934; Sittmann et al., 1971; Thorogood and Ewen, 2006) (Table 4-1). Complete twinning rates for turtle species range from 0.001% to 0.63% per egg, and the percent of females that produce twinning clutches range from 0.4% to 19%. In our ball python dataset the twinning rate per egg was 0.22%, and the percent of females that produced twinning clutches was 2.5%. Therefore, the twinning rates we observed were higher than rates published for avian species, but fell within the ranges published for chelonian species (Table 4-1).

The majority of avian data show that survival of twins is extremely low. Sittmann et al. (1971) and Munro (1965) report survival rates of 0%. For one case in which both the twins did survive, assistance was necessary during hatching (Bassett et al., 1999). In chelonian species the survival rates appear to be higher, but are still reported around 50% or below (Cohen, 1986; Eckert, 1990; Hildebrand, 1938; Messinger and Patton, 1995; Tucker and Janzen, 1997; Yntema, 1971).

Among our 14 sets of twins, only one individual perished during our study (Table 4-2). This individual, from twin clutch number 13, apparently perished in the egg early on in development. All other twins from this study were alive and feeding out to at least three months after hatching, giving us a 97% survival rate for twins in our study.

Table 4-4
Calculation of probabilities for 11 sets of twins with matching color/pattern morphs and sex under the null hypothesis that none of the sets were monozygotic. Color/pattern probability calculations are based on the modes of inheritance published for the associated color and pattern morphs described in Barker and Barker (2006). Probabilities that are at $P < 0.05$ are in bold. Color/pattern morphs as described in Barker and Barker (2006): a = pastel; b = albino; c = axanthic; d = mojave; e = ghost; f = caramel-albino; g = piebald; h = spider; i = calico; j = spotnose; k = lesser; l = pinstripe; and w = wild-type.

TWCL	Sire	Dam	Twin	Color/Pattern Probability	Sex Probability	Combined Probability
2	-	-	-	1	0.25	0.25
3	aa	aw	aw	0.25	0.25	0.0625
4	bw, cw	bb, cw	bb, cw or ww	0.1406	0.25	**0.0352**
5	dd, ew	dw, ew	dd, ee	**0.0156**	0.25	**0.0039**
6	ff	fw	fw	0.25	0.25	0.0625
7	aw, gw	gg	aw, gw	0.0625	0.25	**0.0156**
8	hw, ew	ee, dw	dw	**0.0156**	0.25	**0.0039**
9	iw	ww	ww	0.25	0.25	0.0625
10	jw	aa	aw	0.25	0.25	0.0625
12	kw	dw	dk	0.0625	0.25	**0.0156**
14	lw	aa	aw	0.25	0.25	0.0625
			Totals	**1.3×10^{-10}**	**2.4×10^{-7}**	**3.1×10^{-17}**

Table 4-5

Probabilities of matching microsatellite, color/pattern, and sex for one set of ball python (BP) twins and two sets of carpet python (CP) twins under the null hypothesis that the sets were not monozygotic. Microsatellites MS4, MS5, MS9, MS13, and MS16 are from Jordan et al. (2002). Genotype calculations are based on the modes of inheritance for the associated color and pattern morphs published in Barker and Barker (2006) for the ball python twins, and Julander et al. (2011) for the carpet python twins. Genotypes as described in Barker and Barker (2006): a = spider, and w = wild-type. Genotypes as described in Julander et al. (2011): b = jaguar, c = granite, and w = wild-type.

	MS4	MS5	MS9	MS13	MS16	Color/Pattern	Same Sex	Total
BP sire	454/470	393/409		249/265		aw		
BP dam	454/454	393/393		265/265		ww		
BP twins	454/454	393/393		265/265		ww		
Probability	**0.25**	**0.25**		**0.25**		**0.25**	**0.25**	**9.8 X 10^{-4}**
CP1 sire	414/418	359/359		179/191	362/390	bw		
CP1 dam	430/430	371/371		213/223	366/390	ww		
CP1 twins	418/430	359/371		179/213	362/366	bw		
Probability	**0.25**	**1**		**0.0625**	**0.0625**	**0.25**	**0.25**	**6.1 X 10^{-5}**
CP2 sire	410/410	351/351	194/202	203/219	386/390	cw		
CP2 dam	410/426	351/367	194/202	203/219	386/386	cw		
CP2 twins	410/410	351/351	194/202	203/203	386/390	cw or ww		
Probability	**0.25**	**0.25**	**0.25**	**0.0625**	**0.25**	**0.5625**	**0.25**	**3.4 X 10^{-5}**

Our data also differed from the chelonian data in the rate at which twins were asymmetric, and the proportion of incomplete twinning to complete twinning. While the percentage of twins that were asymmetrical in turtle species have been reported to be approximately 80% (Tucker and Janzen, 1997; Yntema, 1970, 1971), only 6/14 (43%) of the sets of twins in our study were asymmetrical. Perhaps even more surprising is the fact that although bird and turtle studies almost exclusively report higher conjoined twinning rates than complete twinning rates (Byerly and Olsen, 1934; Crooks and Smith, 1958; Hildebrand, 1938; Sittmann et al., 1971; Yntema, 1970, 1971), we did not observe a single occurrence of conjoined twinning in the 6,480 ball python eggs we studied.

Several reports on reptile twinning events have made note that the size of twin-bearing eggs were larger than the other eggs in the clutch. This has been noted among various taxa including avian (Alley and Berry, 2002; Bassett et al., 1999), crocodilian (Blomberg, 1979), lizard (Carpenter and Yoshida, 1967), and colubrid snakes (Singh and Thapliyal, 1973). In addition, Gorzula et al. (1997) mentioned that exporters had alluded that ball python twins usually came from extra-large eggs. Analysis of our twinning data failed to show any significant differences between the sizes of twinning and non-twinning eggs. In fact, P-values for comparisons between twinning and non-twinning eggs in egg mass, egg length, and egg width were all at $P > 0.25$ when analyzed using paired t-tests. Further, the combined masses of sets of twins in comparison to their siblings were also insignificant ($P > 0.50$). Therefore our data do not provide any evidence for differences in egg size between twinning and non-twinning eggs.

Eckert (1990) found that twinning clutches had a significantly higher percentage of yolked eggs in comparison to non-twinning clutches. Tucker and Janzen (1997) found that twinning females were larger in plastron length and laid larger clutches than non-twinning females. Among our data we found differences between twinning and non-twinning clutches to be marginally insignificant for clutch mass ($P = 0.05$) and significant for relative clutch mass ($P = 0.02$) (Table 4-3). Studies on the effects of environmental conditions on the prevalence of developmental anomalies have shown that decreases in temperature or oxygen concentration can significantly increase twinning rates (Newman, 1923; Sittmann et al., 1971; reviewed in Hildebrand, 1938; Landauer, 1967). Newman (1923) reported specifically on how crowding of starfish eggs led to increased twinning rates presumably due to increased CO_2 and decreased O_2 levels among the eggs. We propose that higher proportions of eggs being yolked, larger clutches sizes, and especially larger clutch masses in relation to the size of the female (RCM) in twinning versus non-twinning clutches could possibly lead to a higher rate of metabolism occurring within the given oviductal space of the females, which could lead to higher CO_2 levels among the eggs. Although we do not provide any direct evidence for such a relationship, we submit that the evidence from these larger-scale twinning studies in reptiles warrant further testing to see if such larger clutches/clutch masses do indeed experience more hypoxic conditions.

Twinning in mammals is generally predominated by dizygotic twinning (reviewed in Aston et al., 2008; Gleeson, 1994; although see Blickstein and Keith, 2007 for a notable exception). Among reptiles, more turtle researchers have concluded that the instances of twinning they have observed have been dizygotic (Crooks and Smith, 1958; Yntema, 1970, 1971), but more snake researchers have concluded that they observed monozygotic twinning (Curtis, 1950; Mackness et al., 1998; Manimozhi et al., 2006). All 12 sets of twins for which sex was recorded in our dataset were of the same sex (Table 4-2). One of these sets of twins was of different color/pattern phenotypes that are known to be dominant traits (Barker and Barker, 2006) and thus was determined to be a pair of dizygotic twins. The other 11 sets of twins were all of identical color/pattern morphs. The probability of having 11 sets of same sex pairs without any of the pairs being monozygotic twins would be 2.4 X 10^{-7}, and the probability with both same sex and color/pattern morphs given the known modes of inheritance of the morphs (Barker and Barker, 2006) would be 3.1 X 10^{-17}. Therefore we concluded that both dizygotic and monozygotic twinning had occurred in our ball python population and that likely more of the sets of twins were monozygotic than dizygotic.

We then used microsatellite analysis on DNA from one set of ball python twins and two sets of carpet python twins to determine whether monozygotic twinning had indeed occurred. We present the first molecular data showing monozygotic twinning to have occurred in a non-avian reptile species. Probabilities of the observed microsatellite, same sex, and same color/pattern morph data for the one ball python and two carpet python sets of twins if each pair individually was not monozygotic was 9.8 X 10^{-4}, 6.1 X 10^{-5}, and 3.4 X 10^{-5}, respectively (Table 4-5). Further, under the null hypothesis that none of these three sets of twins were monozygotic the probability would be 2.0 X 10^{-12}.

We conclude that the overall complete twinning rate we observed in our ball python population was comparable to what has been shown in turtle species. Further, we found that twinning was not as disadvantageous in our ball python population as has been reported in turtle species given that the survival rate for ball python twins was 97%. We provide evidence that relative clutch mass is significantly higher for twinning than non-twinning clutches. Also, we observed occurrences of both dizygotic and monozygotic twinning and conclude that monozygotic twinning occurs at a higher rate in our ball python population than dizygotic twinning does. Lastly, we present the first molecular data showing monozygotic twinning to have occurred in a non-avian reptile species.

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CHAPTER 5

SUMMARY

Information regarding reproduction traits of captive reptiles is sparse. Published reports on python species are limited to small sample sizes, and to discussing averages and ranges for reproductive traits. Further, little published information exists that discusses management practices for larger-scale snake reproduction. Breeding programs working with captive populations of reptiles frequently violate the assumptions associated with the use of ordinary least squares (OLS) to calculate breeding genetics values such as repeatability (R), heritability (h^2), and genetic (rG) and phenotypic (rP) correlations. Restricted maximum likelihood (REML) does not have such associated limiting assumptions and lends itself nicely to use with captive breeding data. To date, no studies have used REML to calculate breeding genetics values in any non-archosaurian reptiles. Further, although studies on turtle populations have shown that twinning is generally detrimental to reproductive efficiencies, to date no such analysis has been carried out on any other non-avian reptile species.

Although ball pythons in this study appeared to generally be pulse breeders, clutches were laid during all weeks of the year except the first 3 weeks in January. Further, the above data suggest that the week of the year a ball python female lays her eggs in captivity is heritable and is significantly affected by both maternal and permanent environmental effects. Reproduction throughout the majority of the year in captive ball pythons is in stark contrast to what has been reported to occur in nature. Wild ball pythons in southern Togo, Africa generally lay their eggs during one month of the year.

The fact that female ball pythons can proceed through their reproductive cycles at almost any time during the year in captivity could have important implications for those trying to reproduce other python and snake species. Methods such as follicle palpation and ultrasound may significantly enhance success in reproducing these species in captivity by helping to identify times during which males should be introduced to females for copulation. This would be especially important in situations in which keepers are attempting to breed multiple females with single males.

During the current study, clutch sizes were reduced by the dam when one or more eggs were laid early, or one or more eggs were excluded from the dam's coils during brooding. Although the female mass average from females that produced these reduced clutches was not significantly different from the overall female mass average from all clutches laid in this study, clutch mass was significantly higher in clutches that were laid early, and RCM was significantly higher in both types of reduced clutches. Therefore, clutches were reduced in size when they were large in comparison to female mass (higher RCM), which would potentially lead to females experiencing difficulty in covering the proportionately larger clutches. Also, the hatch rate for clutches that had eggs laid early was significantly lower than the hatch rate for all clutches in this study, but the hatch rate for these same clutches once they were reduced (i.e. not including eggs that were laid early) was not significantly different than the overall average.

Female frequency of reproduction is often discussed among ball python breeders. The suggestion has been made in the past that limiting females to breeding every other year may increase overall production. In our univariate model we did detect a significant effect of consecutive clutches (females laying clutches in two or more consecutive years) on clutch size. However, in the multivariate model this effect was not significant. We were unable to find any convincing evidence from our data to suggest that breeders should purposely limit their females to breeding every other year.

We hypothesized that egg width, and perhaps egg length, could provide an indication of the volume within the oviduct available for eggs, as has been discussed by other researchers. Due to the fact that snakes do oviposit some infertile masses among their clutches that are smaller, we hypothesized that the inclusion of the measurements for these infertile, smaller egg masses would decrease the correlations between these traits and their explanatory factors because these smaller masses would be poor indicators of oviductal space. Further, we hypothesized that averages for egg width and perhaps egg length that included measurements from infertile egg masses would lead to lower h^2 estimates.

Our data provide evidence that it would likely be beneficial for researchers to exclude measurements from infertile egg masses when they are calculating mean egg lengths and widths for use in developing breeding selection programs for ball pythons, and perhaps other snake species as well. Heritability for egg width increased from 0.35 to 0.45 when infertile egg mass measurements were removed, and the correlation between egg width and its explanatory factor MAS was higher when infertile egg masses were removed as well (0.35 versus 0.53). Although the increase in h^2 for egg length was less dramatic (0.47 to 0.48), the correlation with its explanatory factor AGE was also increased (0.08 to 0.13). Given these results, we used the egg length and width means that did not include measurements from infertile egg masses in the multivariate model.

Moreover, we suggest that other researchers working with ball pythons, and perhaps other snake species, do the same.

Maximization of healthy offspring per clutch (HOFF) was deemed to be our main focus in designating selection criteria. Although HOFF, hatch rate (HR), and egg mass (EMAS) were deemed important for use in creating selection criteria, they were not ideal due to lower heritability or genetic variation. Conversely, heritability for CSIZ was high (0.44), and the estimate of genetic variation for CSIZ was among the highest for all the traits (coefficient of variation = 0.26). Also, rG and rP between CSIZ and HOFF were both 0.54. Given the above data, CSIZ appeared to be the most ideal trait to focus on when setting up selection criteria for our captive population of ball pythons.

A trade-off between clutch size and egg size has been shown in many species, including several snake species. For captive ball pythons this trade-off seems to exist as well. The rG estimates between CSIZ and EMAS, EL, and EW were -0.61, -0.71, and -0.29 respectively. The rP estimates between CSIZ and EMAS, EL, and EW were -0.40, -0.63, and 0.14 respectively. Therefore, if selection pressure is applied to produce larger clutch sizes, breeders should pay attention to potential decreases in EMAS because increasingly smaller egg masses will likely decrease hatch rates (rG = 0.42 between EMAS and HR) and potentially lead to decreased HOFF (rG = 0.65 between HOFF and HR).

The majority of twinning data published to date for reptilian species came from bird and turtle species. Most of the avian data show that survival of twins is extremely low, usually at or near 0%. In turtle species the survival rates appear to be higher, but are still reported around 50% or below. Among our 14 sets of twins, only one individual perished during our study. This individual apparently perished in the egg early on in development. All other twins from this study were alive and feeding out to at least three months after hatching, giving us a 97% survival rate for twins in our study.

Among the above data we found differences between twinning and non-twinning clutches to be marginally insignificant for clutch mass ($P = 0.05$) and significant for relative clutch mass ($P = 0.02$). Studies on the effects of environmental conditions on the prevalence of developmental anomalies have shown that decreases in temperature or oxygen concentration can significantly increase twinning. One such report noted specifically that crowding of starfish eggs led to increased twinning rates presumably due to increased CO_2 and decreased O_2 levels among the eggs. We propose that larger clutch masses in relation to the size of the female (RCM) in twinning versus non-twinning clutches could possibly lead to a higher rate of metabolism occurring within the given oviductal space of the females, which could lead to higher CO_2 levels among the eggs. Although we do not provide any direct evidence for such a relationship, we submit that the evidence from larger-scale twinning studies in reptiles warrant further testing to see if such larger clutches/clutch masses do indeed experience more hypoxic conditions.

We present the first molecular data showing monozygotic twinning to have occurred in a non-avian reptile species. Probabilities of the observed microsatellite, same sex, and same color/pattern morph data for the one ball python and two carpet python sets of twins if each pair individually was not monozygotic was 9.8×10^{-4},

6.1×10^{-5}, and 3.4×10^{-5} respectively. Further, under the null hypothesis that none of these three sets of twins were monozygotic the probability would be 2.0×10^{-12}.

The large sample sizes attained in this study have allowed us to study certain aspects of ball python captive reproduction that have not been previously analyzed. Novel data presented in this study provide a foundation for the design of future studies, and for the development of more efficient breeding plans for propagating captive pythons. For individuals and commercial breeders that wish to maximize breeding efficiencies in ball pythons we suggest that they do the following: 1) Utilize palpation and/or ultrasound of follicles in order to ensure females have opportunities to breed with males when ready; 2) Make provisions in breeding procedures to decrease the risk of subjecting eggs to desiccation at any time during incubation; 3) Use clutch size as a primary trait for selection, along with healthy offspring per clutch and hatch rate; 4) As clutch sizes increase, monitor egg masses and adjust selection criteria so as to minimize the negative effects of small egg size on hatch rate and healthy offspring per clutch; 5) Exclude measurements from infertile egg masses when calculating average egg widths and egg lengths for clutches; 6) Perform additional research on twinning in ball pythons and potentially put selection criteria in place that would increase twinning in their populations.

APPENDICES

Appendix A. Permission Letters from Co-Authors

Colette Sutherland
The Snake Keeper, Inc.
811 E 1950 North
Spanish Fork, UT 84660

April 17, 2011

To the Graduate School:

Benson H. Morrill has my permission to use the publication "Effects of captivity on female reproductive cycles and egg incubation in ball pythons (*Python regius*)" that will be published in the June 2011 issue of Herpetological Review, as a chapter in his dissertation.

Sincerely,

Colette Sutherland

Colette Sutherland

Appendix B. Permission to Re-print from Journals

SOCIETY FOR THE STUDY OF AMPHIBIANS AND REPTILES

Publisher of Journal of Herpetology, Facsimile Reprints in Herpetology, Herpetological Review,
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Contributions to Herpetology, *and* Herpetological Conservation

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Gainesville, FL 32627-5638, USA

19 April 2011

BENSON H. MORRILL
Department of Animal, Dairy, and Veterinary Science
Utah State University
Logan, UT 84332

Dear Benson:

This letter is to confirm that your article, “Effects of captivity on female reproductive cycles and egg incubation in ball pythons (*Python regius*)” will be published in *Herpetological Review* (volume 42, number 2, June 2011, pagination not yet determined).

Additionally, permission is granted to reproduce this paper in your dissertation, with any format changes as may be necessary to conform to the requirements of your university. A statement indicating that the paper was published in *Herpetological Review* (citing volume, number, pagination, and year of publication) should be included as appropriate.

Sincerely yours,

Robert Hansen
Editor

CURRICULUM VITAE

BENSON H. MORRILL

555 East 200 North • Logan, UT 84321
(435) 770-5997 (mobile) • (435) 797-3895 (work)
ben.morrill@usu.edu

EDUCATION

- Sky View High School, Smithfield, Utah, 1997–1999, 4.0 GPA, received the Departmental Award for Science

- B.S., Biology, Department of Biology, with honors, minor in chemistry, 3.91 cumulative GPA, Utah State University, Logan, Utah, 2004

- Ph.D., Animal Science: Molecular Biology, dissertation successfully defended 06 May 2011, Department of Animal, Dairy, and Veterinary Science (ADVS), Utah State University, Logan, Utah

CURRENT POSITION

Research Technician III, Dr. Clay Isom's laboratory, August 2010-current, ADVS department, Utah State University, Logan, Utah

FELLOWSHIPS/GRANTS/AWARDS

2004	ASUSU Travel Monies Grant ($450)
2004	USU Undergraduate Research and Creative Opportunities Grant ($700)
2004	USU College of Science Undergraduate Research Grant ($1,000)
2004	Helen B. Cannon Award (USU Honors Department) ($1,000)
2005–2006	USU Vice President for Research Fellowship ($15,000)
2006–2010	USU ADVS Department, Departmental Assistantship ($15,500/year)

2008	CIB Waldron Grant ($500 travel monies)
2008	CIBR Graduate Student Award ($7,000 research monies)
2009	Graduate Student Senate Enhancement Award ($4,000)
2009	1st Place in College, Oral Presentation, USU Intermountain Graduate Research Symposium ($180)

TEACHING AND RESEARCH EXPERIENCE

Teaching Assistant Experience

- Methods in Biotechnology: Molecular Cloning, ADVS 5260; Fall 2006, Fall 2007
- Animal Molecular Biology, ADVS 5280; Spring 2007, Spring 2008, Spring 2009
- Principles of Animal Genetics and Breeding, ADVS 4560; Fall 2008, Fall 2009
- Physiology of Reproduction and Lactation, ADVS 4200; Spring 2010
- Animal Cell Culture Methods, ADVS 5160; Spring 2010

Specific Technical Experience

Extensive Experience in:

- DNA and RNA techniques including extraction and purification, quantification, PCR, qRT-PCR, primer design, probe design, site-directed mutagenesis, microsatellite design, bacterial cloning, sequencing and sequence analysis, bisulfite sequencing and analysis including next generation sequencing data, microarray, and submissions to GenBank
- Use of Geneious and Sequencher bioinformatics software for analysis of genomic, transcriptomic, and epigenetic data (including high-throughput data from next generation sequencing) as well as plasmid engineering for the production of proteins in mammalian cells

- Various cell, tissue, and embryo culture techniques including starting and maintaining primary cell lines

- Quantitative genetics and other biological statistical analyses, and the use of GraphPad Prism 5.0, SAS 9.2, WOMBAT, and ASReml statistical software packages

- Field collection and preservation of small animals for a teaching collection and possible future research

- Reptile reproduction including effective breeding techniques, palpation and other methods for tracking pregnancy

Moderate Experience in:

- Working with Biosafety Level 2 blood born pathogens

- Producing transgenic mammalian cells via viral transduction and transfection using both lipid-based delivery systems and electroporation

- Conducting reproductive hormone treatments in mice to induce superovulation, and collection of oocytes and preimplantation stage embryos

- Assisting in porcine surgeries in which the purpose is to remove oocytes or preimplantation stage embryos

- Collection of immature porcine oocytes

- In vitro maturation of porcine oocytes

- Gas chromatography mass spectrometry (MS) and limited experience with liquid chromatography MS; from sample prep and runs to instrument maintenance and data analysis using Waters machines and software

- Basic veterinary techniques in reptiles, including blood draws and euthanization

- Heat detection in pigs

- Artificial insemination in pigs

Limited Experience in:

- Bovine oocyte collection and IVF embryo production

- Cryopreservation of bovine semen

- Protein techniques including extraction and purification, quantification, western blots, and 2D-gels

Presentations at Scientific Meetings

2004 Poster Presentation, Phylogeography of lowland species of *Bufo* across the eastern end of the Trans-Mexican Neovolcanic Belt, Norman, OK, American Society of Ichthyologists and Herpetologists.

2008 Poster Presentation, Primate sequence length polymorphisms within the Amelogenin and Amelogenin-like genes: Usefulness in sex determination, Kona, HI, Society for the Study of Reproduction.

2008 Poster Presentation, Metabolomic effects of Eastern Cottonmouth venom on mammalian kidney cells, San Francisco, CA, American Society for Cell Biology.

2009 Oral Presentation, Metabolomic effects of pitviper venoms on mammalian kidney cells, Logan, UT, Intermountain Graduate Research Symposium.

2009 Poster Presentation, Metabolomic profile shifts in mammalian kidney cells caused by pitviper venoms, Albuquerque, NM, Venom Week, Third International Scientific Symposium.

2010 Oral Presentation, Generation of induced pluripotent stem cells in livestock species, Logan, UT, Intermountain Graduate Research Symposium.

2010 Poster Presentation, A combined approach to foster undergraduate research in biotechnology and systems biology at Utah State University, Ogden, UT, Council on Undergraduate Research 19th National Conference.

2010 Oral Presentation, Python reproductive traits: Maximizing breeding success and efficiencies, Tucson, AZ, International Herpetological Symposium.

Publications

Mulcahy, D. G., **Morrill B. H.,** Mendelson J. R., 2006. Historical biogeography of lowland species of toads (*Bufo*) across the Trans-Mexican Neovolcanic

Belt and the Isthmus of Tehuantepec. *Journal of Biogeography,* 33, 1889-1904.

Morrill, B. H., Rickords L. F., Shafstall H. J., 2008. Sequence length polymorphisms within primate Amelogenin and Amelogenin-like genes: Usefulness in sex determination. *American Journal of Primatology*, 70, 976-985.

Morrill, B. H., Rickords L. F., Shafstall H. J., 2009. Sequence length polymorphisms within cervid AmelogeninX and AmelogeninY genes: Use in sex determination. *Wildlife Biology in Practice*, 5(2), 89-95.

Morrill, B. H., Rickords L. F., Sutherland C., Julander J. G., 2011. Effects of captivity on female reproductive cycles and egg incubation in ball pythons (*Python regius*). *Herpetological Review*, 42(2), 226-231.

Currently Preparing for Submission in 2011

Morrill, B. H., Rickords L. F., French S. S., Sutherland C., Julander J. G. Quantitative genetic analysis of ball python (*Python regius*) reproduction traits. Will submit to *Journal of Animal Breeding and Genetics*.

Morrill, B. H., Rickords L. F., French S. S., Sutherland C., Julander J. G. Should egg widths and lengths from obviously infertile eggs be removed from quantitative genetic analyses on reproductive traits? Will submit to *Copeia*.

Morrill, B. H., Rickords L. F., Sutherland C., Julander J. G. Twinning in reptiles: evidence of relatively high rates of monozygotic twinning and survival of twins in snake species. Will submit to *Herpetologica*.