Sperm competition in leopardgeckos, *Eublepharis macularius.*

Does mating order affect paternity?

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Fig. 1: Sperm of a Tremper albino leopardgecko, magnified 400x

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Preface

Six years ago I got my first pet reptile, a beautiful wildtype leopardgecko. I told myself that maybe when she had reached adulthood I would a buy male for her. Six months later I owned quite a collection (seven different species of reptiles) and my first baby leopardgecko was born. Since then, breeding leopardgeckos have been one of my biggest passions.

I discovered that the leopardgecko is a many used model animal when it comes to studying different systems in reptiles. And since it is one of the most commercial bred species of reptiles in the world, I figured that basic reproductive systems would have been studied in this species. Many different aspects of reproduction like courting behaviour, incubation temperature and its influence on many things are well studied in leopardgeckos. But the ‘behaviour’ of sperm, a big driving force behind evolution and fitness of a species, never studied. I decided to use my passion for the benefits of science and my study and went to discover it myself. It took quite some time of thinking, acquiring the animals, find a place for them to live and grow and convincing people that the research was really necessary. I am glad however that I did this and it proved to be worth all the ‘trouble’, doing my own research on these ‘weird’ but beautiful animals.

Index:

1. Summary

Many reptiles are promiscuous and therefore mate with more than one partner to ensure higher viability of the offspring. Choosing a partner by selection on dimorphic (sexual) characteristics (as in many birds) is not often seen in reptiles. Sperm competition is therefore believed to be a powerful force that determines the fitness of an individual.

To test whether mating order affects paternity in leopard geckos, so called amelanistic (albino) leopard geckos are used as a model animal. Albino leopard geckos come in three different types and albino juveniles can only be produced when both parents are of the same albino type. This ‘incompatibility’ is used as a tool to determine paternity instead of DNA analysis, because it is much less invasive for the animals. Virgin female Tremper albino leopard geckos are mated with two different males (one Tremper and one Bell albino) and the phenotype of the hatchling leopard geckos is used as a tool to determine paternity; a albino juveniles is sired by the Tremper albino male and a wild type juvenile is sired by the Bell albino male. In total 24 females will be mated with a ‘male-couple’ (= one Tremper albino male and one Bell-albino male) out of a group of 8 males. All the combinations are determined by chance.

The objective of this project is to discover if the order of mating has an influence on paternity. There are three possible scenarios: 1. old (stored) sperm (that has proven itself) is used again to fertilize a new clutch, 2. new (and therefore fresh) sperm is chosen or 3. the two types of sperm are mixed together and it is up to the most viable sperm to win the competition and fertilize the eggs. This is tested by using a one-sample T-test if there is a significant difference in fraction of juveniles that resulted from the first or the second mating. The data suggest that there is no significant difference ($p= 0,679$) and this suggests that mating order does not affect paternity. Also copulation duration did not affect paternity ($p=0.653$) and this contradicts with results found in other studies. The fraction of juveniles sired by a Bell-albino male was however significant lower than the fraction of juveniles sired by a Tremper-albino male ($p=0.016$). This could not be explained by body weight ($p=0.290$) or copulation duration ($p=0.213$). These results and the applications for leopard gecko breeders are discussed.

2. Introduction

2.1 Sexual selection and promiscuous mating

Sexual selection is an evolutionary process that favours the frequency of genes that have a positive effect on reproductive success (Birkhead & Møller, 1993; Birkhead & Pizzari, 2002; Schulte-Hostedde & Montgomerie, 2006). Darwin believed this process to be exclusively pre-copulatory (intra-sexual competition and mate choice) (Stuart-Smith *et al*., 2007), since he thought females showed only monogamous reproductive behaviour (Birkhead & Pizzari, 2002). Almost 140 years later we now know that polyandry (mating with multiple partners) is a very widespread phenomenon that is observed in many different taxa (Jennions & Petrie, 2000; Price & Wedell, 2008).

For a long time it was thought that polyandry was a male driven process with evolutionary advantages for males; if a male mates with multiple partners the more his genes are spread, which increases the male's fitness (Birkhead & Pizzari, 2002). Besides, males typically make smaller parental investments and male's choice should be under less selective pressure (Stuart-Smith *et al*., 2007). This process causes males of many different species to develop ornaments and it is proven that more 'attractive' males have a greater reproductive success (Evans & Simmons, 2007; Hosken *et al*., 2008). Females were believed to be forced to mate with more than one male and had no or little influence on the process (Jennions & Petrie, 2000; Birkhead & Pizzari, 2002).

It is now known that many females actively seek mating and insemination by multiple males, indicating that there is something to gain for the female (Birkhead & Pizzari, 2002; Hosken *et al*., 2008). These benefits are thought to be (1) an increase of viability and fitness of the offspring by selection on traits that improve performance under natural or sexual selection (i.e. pre-copulatory partner choice) (Jennions & Petrie, 2000; Pizzari *et al*., 2003; Hosken *et al*., 2008), (2) reduction of genetic incompatibility of the male and female (Jennions & Petrie, 2000; Price & Wedell, 2008) and (3) the production of more attractive sons, causing an indirect fitness benefit for the female (Jennions & Petrie, 2000; Birkhead & Pizzari, 2002; Evans & Simmons, 2007). By copulating repeatedly (not with different males per se) females can receive nuptial gifts, obtain nutrients from the ejaculate, stimulate ovulation, induce male parental care or increase fertilization success (Hunter *et al*., 1993).

Actively seeking of multiple suitable partners, male-male aggression and sexual dimorphism are examples of pre-copulatory sexual selection (Jennions & Petrie, 2000; Evans & Simmons, 2007; Stuart-Smith *et al*., 2007).

2.2 Multiple paternity in reptiles

Female reptiles often tend to choose to mate with multiple partners, to ensure a high (genetic) quality of the offspring (Olsson & Madsen, 1995; LaDage *et al*., 2008). Female reptiles do not receive nuptial gifts from males and tend to ovulate spontaneously (LaDage *et al*., 2008). Parental care of juveniles is very rarely seen with reptiles (Uller & Olsson, 2008) and it is not proven that female reptiles (as with other squamates) obtain nutrients from ejaculate (LaDage et al., 2008). Promiscuous mating and multiple paternity is observed (and proven) in different species of turtles (Pearse *et al*., 2001; Johnston *et al*., 2006), snakes (Schulte-Hostedde & Montgomerie, 2006) and a high level of multiple paternity is found in different species of lizards (Oppliger *et al*., 2007). The benefit of multiple mating for female reptiles has to be different than the possible benefits that are already mentioned.

Some lizard species (for instance the Sand lizard, *Lacerta agilis*) mate with multiple partners, because the males tend to be infertile a short period of time after emerging from hibernation (the maturation of the spermatozoa is not completed yet) and the reason for promiscuity is in that case of a very practical nature (Uller & Olsson, 2008). Both fertility and fecundity are not influenced by copulating with multiple males, but the reproductive output (number of clutches and clutch mass) does (LaDage *et al*., 2008). According to findings of LaDage *et al* (2008), female leopardgeckos that mated with more than one male, produced more clutches and produced more fertile eggs. These females also invested more in eggs as measured by relative clutch mass, compared to the clutch mass of females that mated with one male. These differences were a result of both an increase in number of times they mated and the number of different males they mated with (LaDage *et al*, 2008).

2.3 Sexual selection and sexual dimorphism in reptiles

Many animal taxa use characteristics of sexual dimorphism or ornaments as a tool to choose the best partner for mating (Olsson & Madsen, 1995). Inter-sexual dimorphism, for instance body size, does occur in different species of reptiles (Stuart-Smith *et al*., 2007), but pre-copulatory partner choice by females, for example the biggest male or the male with the brightest coloration, is poorly documented (Olsson & Madsen, 1995; Cox *et al*., 2003). However, the differences that occur due to inter-sexual dimorphism do play a role in intra-sexual competition (Stuart-Smith *et al*., 2007). Although inter-sexual dimorphism is not very common in reptiles (Olsson & Madsen, 1995; Cox *et al*., 2003), there are some examples that are worth mentioning.

The collared lizard (*Crotaphytus collaris*) for instance shows both body size and colour inter-sexual dimorphism; adult males show a bright green and yellow coloration where the females remain grey and white (Schumacher, 2002). Males also show significant more territorial behaviour. Studies showed that this pattern of sexual dimorphism is influenced by and a product of local differences in selection pressure and environmental conditions. This species is strongly sexual dimorphic and territorial and it is therefore likely that strong intra-sexual selection through resource defence is a major force behind this dimorphism (McCoy *et al*., 2003). Conspicuous coloration also has its costs, which is confirmed by findings that more conspicuous coloration in first-year male *C. collaris* reduces foraging efficiency (and thus growth rate) and therefore has a big influence on future reproductive success, even though this trait is favoured by sexual selection (Baird, 2008). Usually males in this species begin to show their bright coloration after two years (Schumacher, 2002; Baird, 2008) when they are big enough to compete with other males for territory and resources (Baird, 2008).

A very nice example of intra-sexual selection driven dimorphism in reptiles is shown by the side-blotched lizard (*Uta stansburiana*). The differences that occur due to sexual dimorphism, are not used for inter-sexual mate choice, but play a role in male-male competition. The males of this species can be divided in three different colour morphs: orange-throated males, blue-throated males and yellow-throated males. Each morph shows its own mating strategy (and behaviour), with a weakness for one of the other morphs and an advantage over another one, comparable to the rock-paper-scissors game. The orange-throated males are very aggressive and territorial, protecting a relative big territory. The blue-throated males are mate guardians and after mating with a female, these males will chase after every male that tries to approach the female. The yellow-throated males are so called sneakers. They sneak inside the territory of another male and try to mate with the females, all happening very fast and relying on female-mimicry. The orange-throated males possess an advantage over the blue-throated males, but a weakness for the yellow-throated. The blue-throated guard their females against the yellow-throated males, but can be chased away by an orange-throated male. The yellow-throated males will not be able to compete with the blue-throated males, but will win from the orange-throated males when they are chasing after other males, leaving the female off guard (Zamudio & Sinervo, 2000).

A comparable situation is found in the Australian Painted Dragon lizard (*Ctenophorus pictus*). In this species there are three different colour morphs; the red coloured males, orange coloured males and yellow coloured males. The red males are dominant over the yellow males in staged confrontations and the yellow coloured males are superior in sperm competition trials, where orange coloured males are not that common in the wild and therefore no laboratory trials were done with this morph (Olsson *et al*., 2007; Olsson *et al*., 2008). The two studied (red and yellow) morphs show no differences in space use and territory size (Olsson *et al*., 2008). The colouration showed to be a co-dominant polygenetic inheritance model (Olsson *et al*., 2007) suggesting that the different morphs are kept in balance to each other, since in wild populations they showed no difference in the number of offspring sired by red of yellow males despite the difference in mating strategy (Olsson *et al*., 2007); the red males tend to be better mate guarders and the yellow males sire more offspring per copulation (Olsson *et al*., 2007). According to Olsson *et al*. (2007) the red-coloured males "were under negative selection when they dominated the population, which suggests ongoing frequency dependent selection on male colouration" (Olsson *et al*., 2007). This suggests that also in this species, morphological differences are more used for intra-sexual competition, more than pre-copulatory mate choice.

Both inter-sexual and intra-sexual dimorphism can be driven by post-copulatory sexual selection, which means that the selection continues after copulation took place and it could be considered a battle for fertilisation of eggs. Sperm competition is a powerful component in this post-copulatory sexual selection (Birkhead & Pizzari, 2002).

2.4 Sperm competition

Sperm competition occurs when the sperm of multiple males compete to fertilize the eggs of a female (Olsson *et al*., 1994; Schulte-Hostedde & Montgomerie, 2006; Hosken *et al*., 2008). Due to high selection pressure on fertilizing ability, males in many taxa developed different strategies (which can be offensive and defensive) in gaining advantages in this battle (Birkhead & Pizzari, 2002). Both aspects are nicely illustrated by different *Drosophila* species. The ejaculate of males contains seminal substances that deactivates rival sperm already deposited in the female (offensive) and functions as an 'anti-aphrodisiac' which makes the female less attractive for re-mating (defensive) (Birkhead & Pizzari, 2002). Other insects (e.g. Green-veined white butterfly *Pieris napi*) use non fertile sperm to fill the oviducts of the female to delay remating of the female (defensive), giving the fertile sperm more time to fertilize the eggs (Cook & Wedell, 1999). Other examples of offensive insect sperm competition strategies consist of the production of great quantities of small sperm (e.g. the cricket *Gryllus bimaculatus*) (Evans & Simmons, 2007), or displacement of previous stored sperm (Lewis & Austad, 1994). Some defensive strategies are mate guarding and the use of copulatory plugs which can be used as a mechanical and chemical block for sperm of rival males (Foellmer, 2008). Malaria mosquitoes (*Anopheles spp.*) use a gelatinous mating plug that is produced by the males' accessory glands. The plug is inserted in the female after sperm has been transferred and it provides a short-term barrier (for 24 to 48 hours) against additional copulations. Long-term inhibition of female receptivity can be accomplished by a substantial quantity of ejaculate in the spermatheca (Yuval, 2006). Mating plugs do not only exist of component produced by the genital glands of the male, but some species (e.g. the orb-web spider, *Argiope aurantia*) males use broken genital parts or their entire (dead) body as a mating plug (Foellmer, 2008). Also in higher taxa, many different strategies of sperm competition occur.

In different kinds of externally fertilizing fish, measures of sperm quality as swimming speed and percentages of motile sperm seem to regulate fertilizing success (Birkhead & Pizzari, 2002). In guppies (*Poecilia reticulata*, a species with internal fertilisation) the swimming speed and sperm viability also has a great influence on paternity (Evans & Simmons, 2007). Sperm size also has an influence on paternity in many species, however there are some contradictions on this matter, i.e. some studies in mammals and birds show no correlation between sperm size and fertility success, others show that shorter sperm has an advantage and again other studies show that larger sperm has an advantage depending on species (Birkhead & Pizzari, 2002).

A remarkable sperm competition related behaviour is observed in the Small-mouthed salamander (*Ambystoma texanum*). This species mates in large groups, where the males place their spermatophores on the pond substrate and de females have to find and obtain sperm from them (Harris, 2008). Harris (2008) found that males deposit their own spermatophores on top of those of rival males (a phenomenon called spermatophore capping behaviour). It is hypothesised that they do so in order to make the sperm of the rival male less easy available for the female and thus increasing their own chance in fertilizing the female (Harris, 2008).

In big mammal carnivores, Iossa *et al*. (2008) found that in species with shorter mating seasons, males should invest relatively more in spermatogenic tissue (i.e. bigger testes relative to their body weight) because of a higher demand for sperm production. Also the type of ovulation (spontaneous or induced ovulation) has an influence on testes size, meaning that in species that show spontaneous ovulating species, the males show a relative higher number of spermatozoa per volume ejaculate (Iossa *et al*., 2008). In other species the opposite is observed; once the intensity of sperm competition becomes very high, the probability of fertilization of each male is reduced. Males therefore tend to invest less in the number of sperm and more carefully allocate the sperm available to maximize the number of females they can fertilize (Birkhead & Pizzari, 2002). This phenomenon is observed in crabs, the blue headed wrasse (Birkhead & Pizzari, 2002) and the fowl (Pizzari *et al*., 2003).

2.5 Sperm competition in reptiles

In reptiles spermatogenesis can be energetically expensive, which indicates there are costs to produce high quality sperm (Schulte-Hostedde & Montgomerie, 2006). In the Nothern Watersnake (*Nerodia sipedon*) a large variation on different sperm traits exists, such as motility and the number of sperm per volume ejaculate. This species is highly promiscuous (mating occurs in groups) and qualitative sperm traits seem to play an important role in sperm competition in this species (Schulte-Hostedde & Montgomerie, 2006). It is possible that in other species of reptiles these type of traits also are important.

Besides increasing the number of sperm per volume ejaculate, it is also possible to deposit more ejaculate into the female. In the agamid lizard *Ctenophorus fordi*, Olsson observed that males mated longer with a female if they witnessed the same female copulating with another male first (Olsson, 2001). These results suggest that males may prolong the copulation to become more competitive by transferring more competing spermatozoa when the risk of sperm competition is increased (Olsson, 2001). The same behaviour is observed for the Iberian Rock lizard (*Lacerta monticola*) where males prolonged copulation with a female that already mated with another male (Moreira & Birkhead, 2004). The duration time of the copulation does not seem to affect the fertility, fecundity and maternal investment in egg mass in leopardgeckos (LaDage et al., 2008) and therefore prolonging copulation time might only raise benefits for males.

Another way to increase success in sperm competition is to prevent rival sperm from entering the female. This can be accomplished by raising mechanical barriers (e.g. mating plug (Moreira & Birkhead, 2003)) or by different behavioural traits such as mate guarding. This phenomenon is observed in many different species of reptiles e.g the scincid *Eumeces laticeps* (Cooper & Vitt, 1997), the lacertid *Lacerta monticola* (Moreira & Birkhead, 2003) and also the gecko *Ptyodactylus guttatus* (Johnston & Bouskila, 2007). Besides mate guarding is a species specific behaviour, it can be an intra specific variable behaviour, where only the males of a particular morph show mate guarding behaviour (see “Sexual selection and sexual dimorphism in reptiles”) (Zamudio & Sinervo, 2000; Olsson *et al*., 2007; Olsson *et al*., 2008).

As with many insects (Foellmer, 2008), the use of mating plugs also occurs in reptiles (Moreira & Birkhead, 2003). In the Iberian Rock lizard (*Lacerta monticola*) however, these plugs do not seem to have a positive effect on insemination success of the first male mated with the female (Moreira & Birkhead, 2003; Moreira *et al*., 2007), since males seem to be able to remove or loosen a plug deposited by a rival male (Moreira & Birkhead, 2004). It is thought that the plugs serve another purpose by creating a positive pressure in the cloaca of the female that pushes sperm forward (Moreira & Birkhead, 2003). It is also possible that the plugs prevent sperm from leaking out of the female or that the plugs prevent the female to eject sperm (Moreira & Birkhead, 2003) as seen with fowls (Pizzari & Birkhead, 2000).

2.6 Amphigonia retarda

The ability of females to store sperm and keep it fertile for longer periods of time is called *amphigonia retarda* (Seufer *et al*., 2005). This phenomenon is documented for many species (Uller & Olsson, 2008) and the duration varies between reptiles (maximum of 7 years) and birds (maximum 117 days). Mammals show both very short (less than 1 day) and relative long periods (maximum 198 days) (Birkhead & Møller, 1993). Whether a species is oviparous or viviparous determines the ability and duration of sperm storage by females (Birkhead & Møller, 1993; Girling *et al*., 1997). Practically all birds and many reptiles developed specialized ovarian structures for storing sperm (Birkhead & Møller, 1993; Sever, 2002). These specialized structures (see figure 2. “t” being the so called transitional region: the actual site of sperm storage in a gecko *Hemidactylus frenatus*) are also found in different species of geckos (Murphy-Walker & Haley, 1996; Girling *et al*., 1997).

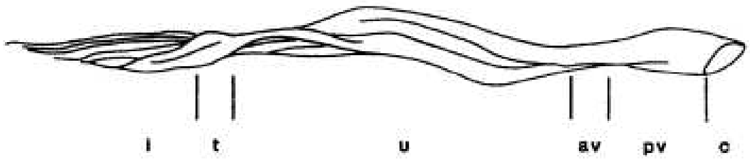


Fig. 2: Schematic of the oviduct of the gecko *Hemidactylus frenatus*: i = infundibulum (3.0 mm), t = transitional region (0.5 mm; site of sperm storage), u = uterine region (5.0 mm), av = anterior vagina (0.5 mm), pv = posterior vagina (1.5 mm), c = cloaca. (Murphy-Walker & Haley, 1996)

Therefore different species of geckos are capable to store sperm and use it to fertilize their eggs, sometimes for many years after copulation took place (Murphy-Walker & Haley, 1996; Girling *et al*., 1997). It is believed that this phenomenon increased the reproductive success (and therefore fitness) of females when males are scarce, for example when a new habitat is colonized (Murphy-Walker & Haley, 1996). LaDage & Firkin found that the reproductive physiology of female leopardgeckos is not affected by non-tactile male cues and it is believed that female geckos do not need to alter the timing of ovulation due to the ability of sperm storage (LaDage & Ferkin, 2008). Figure 3 shows an electro-micrograph of sperm (Sp) stored in the oviductal lumum (Lu) of the infundibulum of the Black swamp snake, *Seminatrix pygaea*. The arrow named “Es” points at spermatozoa that are embedded in the wall of the oviduct.

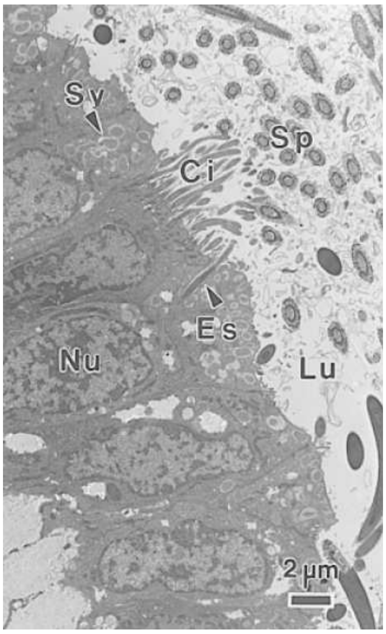


Fig. 3: Electro-micrograph of sperm embedded (ES) in the oviductal lumen of the posterior infundibulum of the Black swamp snake, *Seminatrix pygaea* (Sever, 2002)

The ability to store viable sperm is thought to be a very powerful tool in sperm competition (Uller & Olsson, 2008). It also gives rise to another phenomenon called selective fertilisation of cryptic female choice (CFC), where females ‘choose’ to use the sperm of one particular male in favour of another male. The process takes place after mating and is called ‘cryptic’ because it takes place inside the female. The idea of cryptic female choice in reptiles fits well with the observation that pre-copulatory mating choice is rarely seen in reptiles (Olsson & Madsen, 1995; Uller & Olsson, 2008). A good example of cryptic female choice in reptiles is observed in the side-blotched lizard, *Uta stansburiana*. Here females showed to use the sperm of larger males to produce male offspring and the sperm of smaller males to produce female offspring (Calsbeek & Sinervo, 2004). This indicates that females use the presence of the sex-chromosomes (Calsbeek & Sinervo, 2004) although other studies suggest that the use of other components in the ovarian fluids have an influence of sperm viability, swimming speed and other qualitative traits (Rosengrave *et al*., 2008).

2.7 Leopardgeckos

The leopardgecko (figure 4), *Eublepharis macularius* (Reptilia, Sauria, Gekkonidae), is a middle sized nocturnal lizard which inhabits the rocky savannahs of India, Pakistan, Afghanistan and Iran (Seufer *et al*., 2005). It is one of the most kept pet reptiles in the world, because it’s known to be the perfect species to begin the terrarium hobby with (Vosjoli *et al*., 2005). Because the animal does not grow that big (up to 25 cm in total length) and is relatively easy to maintain and breed, it is also a many used species in research on reptiles (Vosjoli *et a*l., 2005).

Figure 4: adult wild type leopard gecko female

2.8 Reproduction

Leopardgeckos are geckos that use bisexual mating in order to reproduce; parthenogenesis is never described for this species (Rösler, 2005; Vosjoli *et al.*, 2005). The difference between male and female leopard geckos is quite easy to see. The males develop two ‘sacks’ at the base of the tail to hide the hemipenises and have a V-shaped row of pre-anal pores, which produces a wax like substance which contain (among other substances) pheromones (Seufer *et al.*, 2005; Vosjoli *et al.*, 2005). Besides these differences, males tend to grow larger (110-158 mm) than females (109-127mm) (Seufer *et al.*, 2005). Sexual dimorphic differences in colouration and pattern of the skin, or any other physical appearances (such as ornaments) are not documented for leopard geckos (Vosjoli *et* al., 2005).

When the females reach a weight of approximately 35 grams, they become sexually mature (Seufer *et al.*, 2005), but they tend not to show receptive behaviour already at this stage. When the females reach a weight of 40 – 45 grams, reproductive behaviour is shown (Vosjoli *et al.*, 2005). This behaviour exists of standing still and a lift of the tail, so the male can reach the cloaca of the female. The gestation period depends on both internal and external factors such as temperature (Rösler, 2005) or state of nutrition of the female (Seufer *et al.*, 2005) and takes usually 14 to 28 days (Seufer *et al.*, 2005; Vosjoli *et al.*, 2005). The eggs (two eggs per clutch) are laid by burying them in a moist substrate (Rösler, 2005) with a suitable incubation temperature of 26 – 32 °C (Bragg *et al.*, 2000) and will hatch after 45 – 90 days (Rösler, 2005).

2.9 Effects of incubation temperature on leopard geckos

Leopardgeckos are known to use temperature dependent sex determination (TSD) to determine the sex of the offspring (Viets *et al.*, 1993); 26°C produces nearly 100% female offspring, 32,5°C produces mainly males and 34°C results in females again (Viets *et al.*, 1993). It is believed that because of TSD, female leopard geckos carefully choose a suitable nesting site with an optimal temperature, to ensure diversity in offspring sex (Bragg *et al.*, 2000). Incubation temperature does not only affect the sex of the offspring, but also the incubation time (Vosjoli *et al.*, 2005), different behaviours of the animals when the animals reached adulthood (Flores *et al.*, 2004). For instance, leopard geckos that are incubated at a higher incubation temperature tend to be more aggressive than animals incubated at a lower temperature (Flores *et al.*, 2004). Furthermore, incubation temperature affects melanin and other pigment’s production in leopard gecko’s skin; animals incubated at a higher temperature are more bright coloured than animals incubated at a lower temperature (Seufer *et al.*, 2005; Vosjoli *et al.*, 2005). This difference in colouration is most striking in the so called albino or amelanistic leopard geckos (Gamble *et al.*, 2006).

2.10 Albinism in leopardgeckos

Colours and patterns in leopardgeckos are created by a combination of three different pigment cells that are formed in the skin of the animal; melanophores that contain melanin (brown / black), xanthophores (and erythrophores) that contain pteridines (red) or carotenoids (yellow) and iridophores that contain crystallized purines that create different colours due to reflection of light (Gamble *et al.*, 2006). One colour mutation that is not rare with leopardgeckos is amelanism (albinism) and is caused by a lack of black pigment; melanin (Vosjoli *et al.*, 2005). In contrast to many other albino animals, albino reptiles are very colourful, since the other two types of pigments are present in the skin of the animals (Seufer *et al.*, 2005; Gamble *et al.*, 2006). Another difference with other albino animals is that many albino reptiles lack the typical red eyes (Vosjoli *et al.*, 2005). Pigmentation of the skin and that of the eyes are clearly not linked in many reptiles (Vosjoli *et al.*, 2005; Gamble *et al.*, 2006).

In leopardgecko three different types of albinos are known and all three are named after the breeder that commercialised the particular albino strain; Tremper albinos (figure 5), Bell albinos (figure 6) and Rainwater (Las Vegas) albinos (figure 7) (Vosjoli *et al*., 2005). The three strains of albinos are all caused by one recessive gene (Vosjoli *et al*., 2005) that causes a blockage in the production of melanin. One of the most common causes of amelanism in animals is the lack of the enzyme tyrosinase, a catalyser in the melanin production cascade (Gamble *et al*., 2006). All three albino strains of leopard geckos proven to be tyrosinase positive however, which means that the lack of melanin is caused by a non-tyrosinase-dependent defect in the melanin pathway or transport of precursors or products of the melanin cascade (Gamble *et al*., 2006). The Bell albinos however are darker (in most cases), and this is caused by a higher activity of tyrosinase than in both other albino strains (Gamble *et al*., 2006; Vosjoli *et al*., 2005). The three different strains of amelanism in leopard geckos are caused by one simple recessive gene, but a different gene per albino strain. Therefore different albino types are not compatible which means that an albino leopardgecko can only been produced when both parents are of the same albino strain (Vosjoli *et al*., 2005; Gamble *et al*., 2006).

For instance:

Tremper albino x Tremper albino →
Tremper albino offspring (pink and yellow, figure 8)

Tremper albino x Bell albino →
Wild type offspring (black and yellow, figure 9)

For the correct understanding of the experimental set-up, it is important to understand and remember that the different strains of albinos are not compatible for the albino trait. The difference between the (Tremper) albino and wild type adults (see figures 4 & 5) and (Tremper) albino juveniles and wild type juveniles are quiet clear and obvious (compare figures 8 & 9).


Figure 5: adult Tremper albino male


Figure 6: adult Bell albino male


Figure 7: adult Rainwater albino


Figure 8: Tremper albino juvenile


Figure 9: Wild type juvenile

The leopardgecko is also known for its ability to store sperm (see 1.6: “Amphigonia retarda”) and use the sperm to fertilize up to 5-8 clutches (Vosjoli *et al*., 2005, personal experience). It is also believed that stored sperm can survive the hibernation period and subsequently is able to fertilize multiple clutches (Vosjoli *et al*., 2005; Murphy-Walker & Haley, 1996; own observation). Promiscuity is also documented for different species of Eublepharids and also leopardgeckos (Seufer *et al*., 2005; LaDage *et al*., 2008; own observation) and therefore sperm competition is very likely to also occur in this species. Whether the female has a part in sperm competition (cryptic females choice) is not known; is she able to choose which sperm she uses to fertilize the eggs? Different scenarios are possible; the FIFO method (First In First Out) is a possibility, especially if the sperm proved itself to be good and fertile sperm. Besides that it is also possible that when a female mates with another male, that the fresh sperm is preferred over the stored sperm (fresh is better). A third possibility is that the two kinds of sperm are mixed together and that it’s up to the sperm itself to compete with one another inside the female. Since multiple paternity in reptiles is not an unknown phenomenon ((Pearse *et al*, 2001; Schulte-Hostedde & Montgomerie, 2006; Oppliger *et al*. , 2007), this could be a good possibility. My aim of this research is to determine whether the order of mating has an influence on the paternity of the offspring in leopardgeckos, *Eublepharis macularius*.

3. Experimental approach

3.1 How to determine paternity?

Modern technology enables us to compare different DNA samples and by using DNA fingerprinting, paternity in animals is relative easy determined. For this experiment however, DNA fingerprinting is not used for several reasons; 1. the costs of the experiment would be significantly higer, 2. for the collection of DNA samples, blood has to be drawn or a small piece of the tail has to be taken from the animals and 3. the incompatibility for the albino phenotype (see: chapter 2.10) makes is unnecessary. So after two males mated with the same female, the phenotype of the juveniles is used to determine paternity, in other words: ‘which sperm won the competition’. In this experiment one virgin female leopardgecko is mated with one male and after laying the first clutch of eggs, she copulates with a second male. Since leopard geckos are able to store sperm the first clutch is used to check if the first type of sperm (that of male no. 1) is inside the female. The eggs can be candled and therefore show if they are fertilized or not. Since all females are virgins before the first mating, the first clutch is 100% a product of the mating with male no.1, in other words: the sperm of the first mating is present in the female. After the second mating (with a different male) it is assumed that two different types of sperm are present in the female. All the other clutches are the true results of the experiment, since the paternity of these juveniles determine the outcome of the sperm competition question. Figure 10 clarifies the treatment.

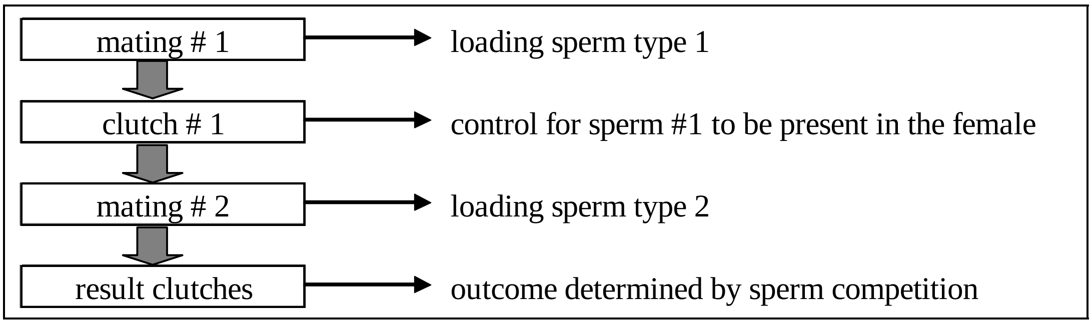


Figure 10: Treatment that is used to determine sperm competition

For this experiment the incompatibility of the different albino strains (see 1.10 Albinism in leopardgeckos) is used as a tool to determine paternity. Twenty four Tremper albino female leopard geckos, four Tremper albino males and four Bell albino males are used. As stated earlier, one female is mated by two males; one Tremper- and one Bell albino. When an albino juvenile hatches (figure 8), it is certain that the Tremper male is the father and when a wild type hatches (figure 9), the Bell albino is the father. Which males mate with which females is determined by chance; first all different combinations of the eight were made and by using the RANDOM-function in Microsoft Excel 2002 these ‘male-couples’ were put in random order. All females were divided in two groups (again using the RANDOM-function). These groups were randomly divided and combined to the different ‘male-couples’. The table that can be found on appendix 1 is the result of the process.

3.2 Health check

In this study twenty four females and eight males are used. All the females are of the Tremper albino type. Four males are also of the Tremper type and the other four males are of the Bell albino type. All the animals are daily checked for their physical condition and pregnant females are very closely monitored. The weight of the animals reflects the animals overall state of health and therefore all animals are weighed once a week.

3.3 Housing

Figure 11: Decoration of one female's cage. Left the moist hide, in the middle meal worms an on the right a water bowl.

All the female animals are housed solitary in so called sweater boxes which measure 45*25*17 cm (l*w*h) as shown in figure 11. The boxes are very easy and clean in maintenance and are semi transparent to ensure light entering the boxes and the animals are not to much disturbed by movement outside the box. The boxes are covered with sand as a substrate and a hiding place filled with moist coco peat is placed in the boxes. These hiding containers also function as an oviposition- / nesting side. The boxes are heated by an under tank heater to ensure the temperature at one end of the box is 30-32°C. The temperature at the other side of the tank (which is thus unheated) will be just above room temperature (22-25°C). The males will be housed in sweater boxes that measure 60*40*18 cm (l*w*h), because the females will be mated in these boxes also. For an unknown period of time, the boxes will therefore contain two animals, that is why the 'male boxes' are bigger. As a substrate also sand is used and the boxes are heated in the same way the female boxes are too. The moist hiding place is replaced by another hiding place and moisture of the substrate is provided by spraying water. All the boxes contain a water dish.

3.4 Feeding

The diet of the animals consists of mealworms (*Tenebrio molitor*) which are ad libitum provided in a shallow dish. Furthermore the diet consists of different kinds of insects, such as brown crickets (*Acheta domestica*), field crickets (*Gryllus campestris*) and Jamaican field crickets (*Gryllus assimilis*), locusts (*Locusta migratoria* and *Schistocerca gregaria*). Besides that, the larvae of *Pachnoda marginata* and waxworms (*Galleria mellonella*) are offered to the animals. The mealworms are powdered with pure calcium carbonate to provide in the calcium need of (pregnant) females and (growing) males. The other meals are powdered with a vitamin supplement (CALVIREP® vit.D3+, by Namiba Terra) or a mineral supplement (MINERALL®, by Sticky Tongue Farms) to provide in the high demand for vitamins and minerals. Besides this, a dish with pure calcium carbonate will be placed in the box ad libitum, so the animals can lick the dust in its pure form if the want to. All boxes contain a water dish that will be cleaned daily.

3.5 Copulations

The 'loading of the sperm' (see chapter 3.1) is done by the most natural and non invasive way possible: by copulation. In order to copulate, the females are placed in the cage of the male that is appointed to her (see 3.1 and appendix I). Every male is allowed to mate with a female once. The duration of the copulation is measured and noted. After copulation the female is removed from the male's cage and placed in her own again. Females are allowed to copulate if they reached a weight over 40 grams. When the animals are house separately, males tend to be very eager to copulate and copulation will usually take place within minutes after putting the animals in the same cage.

3.6 The eggs


Figure 12: Leopard gecko eggs

Every day the hides of the females that are close to oviposition, will be checked on the presence of eggs. Every clutch will be weighed before it is put in the 'au bain-marie incubator' which is constructed by putting a water reservoir that is heated by an aquarium heater in a refrigerator. This is much cheaper than a professional incubator and it was not possible to move an incubator (in another building of the university) into the same room as the animal were taken care of. The professional incubator is therefore not used for practical reasons and this 'au bain-marie' incubator proved to incubate leopard gecko eggs very well for many years (own experience). The substrate of incubation will be moist vermiculite (figure 12), which is known for its ability to obtain water. Incubation temperature will be 30 °C and will last for approximately 50-60 days before hatching. In total up to 100 - 150 eggs were expected.

3.7 The juveniles

When the juveniles of one clutch hatched, they were housed together in a non-transparent box which measures 25*18*7 cm (l*w*h) with a transparent top. In the box a hide and a moist piece of (kitchen) paper were available to the young. The boxes were heated by the same mats that were used or the adults and light can enter the box by the top. The juveniles were fed three times a week, with the same food items the adults were fed (size is adjusted to the size of the juveniles). After two weeks the animals were moved to sweater boxes which measured 60*40*20 cm where they were housed together with other juveniles of the same size (not more than 8 juveniles per cage). When the juveniles proved to be good and healthy animals (they eat, defecate and shed properly), the animals were sold to other (amateur) breeders for breeding purposes or as a pet.

3.8 Embryos

Due to an accident with the incubator (see: 5.2 "Loss of results" on page 22), a lot of eggs were lost. The embryos were removed from the eggs and weighed. The results were put in a graph using Windows XP, Excel 2002. Some of the embryos were preserved using 90% alcohol and were photographed later. All the results and photographs can be found at chapter 4.3 "Embryonic development" on page 17.

3.9 Statistics

To determine if mating order affects paternity, a one-sample T-test is done. The fraction of juveniles that is a result of the second mating will not be significantly different from 50% (P=0.5). The tested hypothesis is:

H_0: P = 0.5 H_a: P $\neq$ 0.5

The same one-sample T-test is done to determine if the juveniles that were sired by a Tremper-albino or a Bell-albino. Here, "P" is stated to be the fraction of juveniles that was sired by the Bell-albino. Again, if there is no difference, than "P" will not be significantly different from 0.5.

To test for the relation between albino-type and copulation duration, I used an independent-samples T-test. For the T-test, the mean copulation duration and the males albino type were used as variables.

To test for the relation between copulation duration and paternity, I used an independent-samples T-test. For the T-test, copulation duration and the “winner” of the copulations were used as variables.

To test for differences in weight over time (duration of the experiment) between the Tremper-albino males and Bell-albino males, I used an ANOVA for Repeated Measurements (rmANOVA). Using SPSS, I made a graph to show the mean body weight per albino type.

4. Results

4.1 Mating order

The one-sample T-test showed that the fraction of juveniles resulted from the second mating (0.4464 ± 0.1265; mean ± standard error) did not differ significantly from the fraction of juveniles resulted from the first mating (t= -0.424; df= 13; p= 0.679). The complete data is found in appendix II and the SPSS output can be found in appendix IIIa.

4.2 Tremper-albino vs. Bell-albino

The one-sample T-test showed that the fraction of juveniles sired by a Bell-albino male (0.2207 ± 0.1011; mean ± standard error) is significantly lower than the fraction of juveniles sired by a Tremper-albino male (t= -2.764; df= 13; p= 0.016). The complete data is found in appendix IIa and the SPSS output can be found in appendix IIIa.

4.3 Albino-type and copulation duration

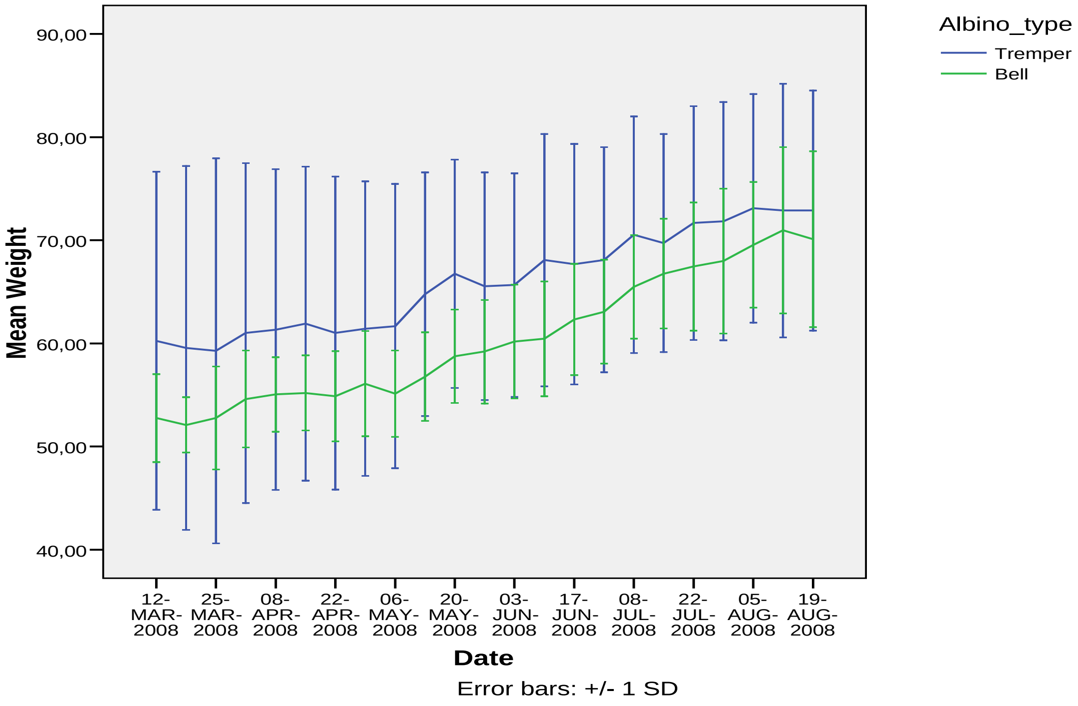
The independent samples T-test shows that the copulation duration between Tremper (1)-albino males does not significantly differ from the Bell (2)-albino males (t= 1.277; df= 26; p= 0,213). The complete data can be found in appendix IIc and the SPSS output can be found in appendix IIIa.

4.4 Copulation duration and paternity

The independent samples T-test shows that the relation between copulation duration and paternity was not significant (t=-0.455; df=26; p=0.653). The SPSS output can be found in appendix IIIb. The complete data can be found in appendix IIb

4.5 Albino-type and weight

According to the rm-ANOVA, there was no significant difference between the body weight of the Tremper-albino males and the Bell-albino males over time (F=1.483; df=1; p=0.290). The complete data can be found in appendix IIb and the SPSS output can be found in appendix IIIb. Graph 1 shows the mean body weight per albino type over time.



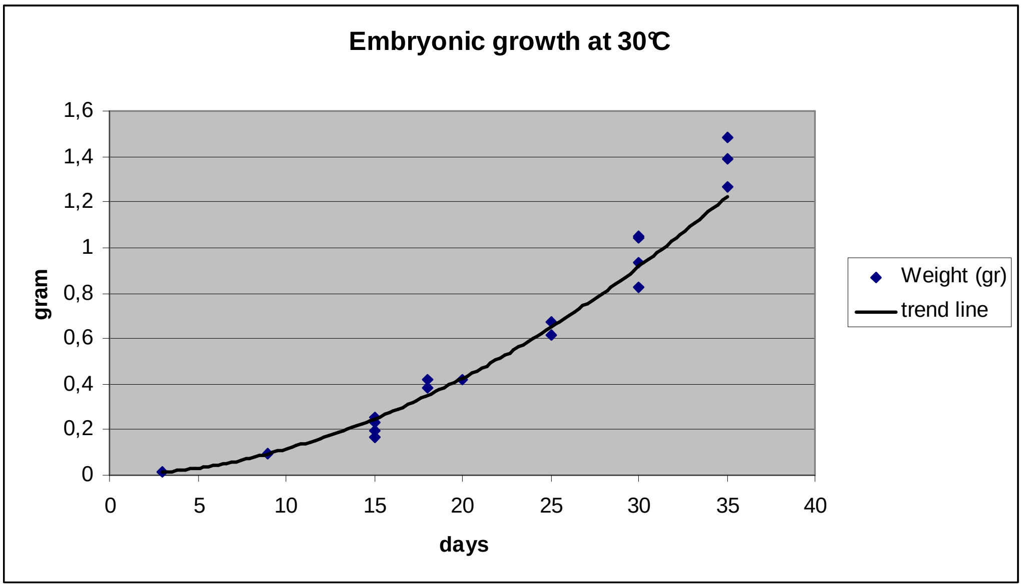
Graph 1: Mean weight of the males per albino-type over time.

4.3 Embryonic development

Table 1 shows the weight (in grams) of leopardgecko embryos after a number of days incubated at 30°C (± 1,5°C). The same results are shown in graph 2 and a trend line is added to indicate grow rate.

# days	Weight (gr)
3	0,0156
9	0,0947
9	0,0949
15	0,1635
15	0,2517
15	0,1681
15	0,2288
15	0,192
18	0,4185
18	0,3829
20	0,4182
25	0,6725
25	0,6159
30	1,0479
30	1,0461
30	0,9351
30	0,8238
35	1,2697
35	1,3892
35	1,4861

Table 1: Age and weight of embryos that died inside the egg after the incubator accident.



Graph 2: Age and weight of embryos that died inside the egg after the incubator accident.

The following photos (figures 13-21) on the next pages are leopard gecko embryos at the mentioned number of days, incubated at 30°C (± 1,5°C).

Figure 13: 3 days

Figure 14: 12 days

Figure 15: 18 days

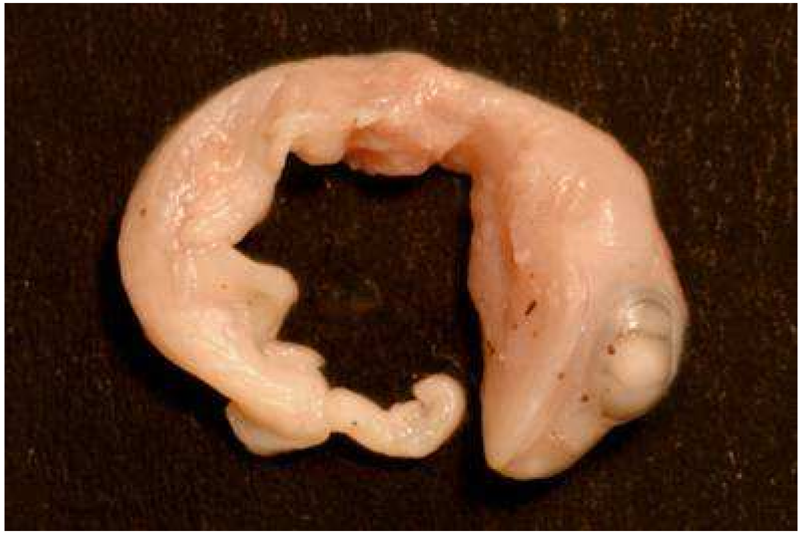


Figure 16: 20 days

Figure 17: 30 days

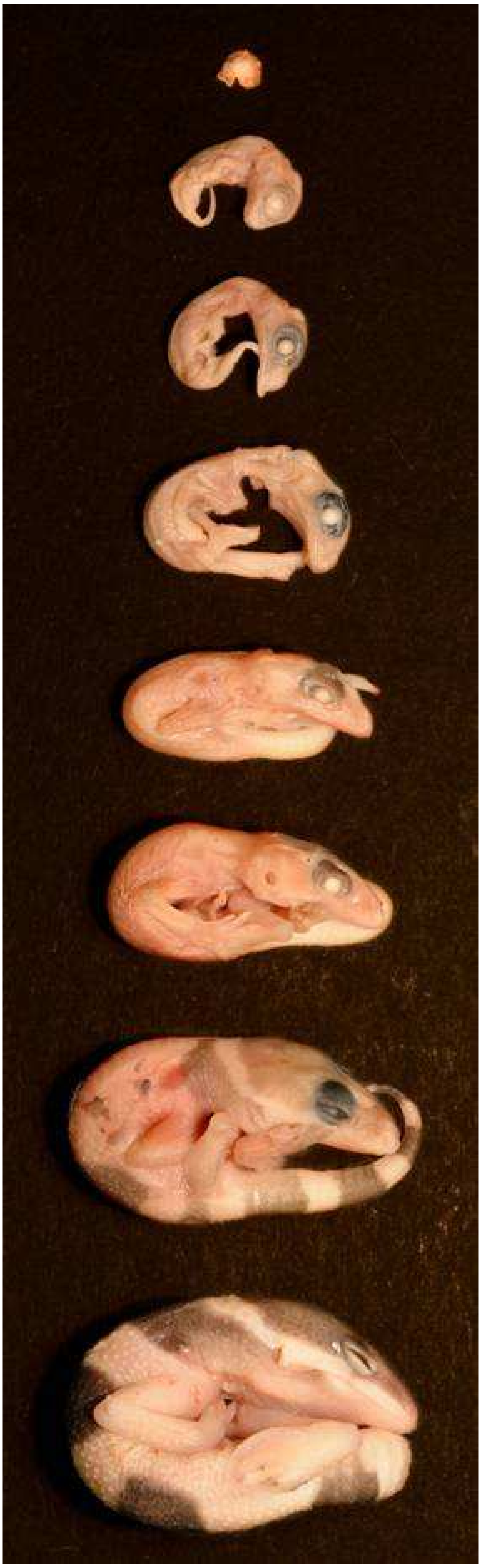
Figure 18: 35 days

These foetuses are of an unknown age, because the first period of incubation was not carried out in the same incubator and thus under different circumstances. It is clear though that these foetuses are the next stages of embryonic development in leopard geckos.

Figure 19: Unknown age 1

Figure 20: Close to hatching

Figure 21: A line up of embryonic development



3 days

12 days

18 days

20 days

30 days

35 days

?

Close to hatching

5. Discussion

5.1 Mating order

According to the results (see: 3.1) there is no significant difference between the fraction of juveniles that is a result of the first mating or the second mating. This indicates that mating order does not affect paternity. This could be explained by the fact that the sperm the female receives from both copulations is mixed or at least stored together in the oviducts of the leopardgecko. Mating with multiple males increases fertility and fecundity in leopardgeckos (LaDage & Ferkin, 2008; LaDage *et al*., 2008) and it is therefore not a surprise that multiple paternity also occurs with this species. This hypothesis is powered by the fact that out of the 25 clutches that hatched, three of them were so called 'mixed clutches' (see figure 22). This means that one juvenile was conceived by the male of the first mating and the other juvenile by the male of the second mating (see appendix IIa, table 2). The fact that this occurred with three different females (female # 1, #10 and #17), each mated with different males, is strong (qualitative) evidence, that multiple paternity is not only possible with leopard geckos, but can occur on a regular basis. In addition the 'mixed clutches' show that 'old sperm' (obtained from the first mating) is still very capable of fertilization and is strong enough to compete with 'fresh sperm' (obtained from the second mating) and that 'freshness' of the sperm has no effect on paternity. Multiple paternity supports the finding that not one type of sperm is preferred by female leopard geckos, but the sperm of two separate matings is mixed during storage. Aspects in the mating behaviour of female leopardgeckos support the hypothesis; first female leopardgeckos readily mate with previous mates as well with novel males (Vosjoli *et al*., 2005; LaDage *et al*., 2008) and second, female leopardgeckos tend to mate more than once before oviposition (personal observations, LaDage *et al*., 2008). Multiple paternity is observed in many species of reptiles (Pearse *et al*., 2001; Johnston *et al*., 2006; Schulte-Hostedde & Montgomerie, 2006; Oppliger *et al*., 2007) but to my knowledge, this is the first real proof that multiple paternity occurs with leopardgeckos.

Figure 22: One of the mixed clutches that hatched. The left juvenile is clearly an albino and the right one clearly is a wild type leopardgecko.

5.2 Bell vs. Tremper albinos

The results show that the fraction of juveniles sired by Bell-albino males is significantly smaller than the fraction of juveniles sired by Tremper-albino males (p=0.016) suggesting that albino phenotype affects paternity. However, the data show there is no difference in copulation duration between Bell-albino and Tremper-albino males (p= 0.213), so this is not the explanation. Besides this, there was not a fixed order in which the males mated with the females (see Appendix 1) and reciprocal crossing were performed. Therefore mating order and albino type were not linked so this also can not be an explanation. However, different breeders of leopard geckos, both amateur as professional, claimed that Bell albinos would be ‘weaker’ than Tremper albinos; they would grow slower, show more health problems and would be less productive than Tremper albinos. Although Bell albinos and Tremper albinos suffer from the same phenotypic ‘handicap’ (lack of melanin in the skin), there is a slight difference (Vosjoli *et al*., 2005; Gamble *et al*., 2006; see figure 6). This slight difference in skin coloration is due to variation in tyrosinase activity in the skin between Bell albinos and Tremper albinos (Gamble *et al*., 2006). There is however no scientific evidence that this difference in tyrosinase activity has an influence on the growth, health or fitness of a Bell-albino individual. The males of both albino types are raised under the exact same conditions (temperature, space, food, etc) and I did not notice any difference in mean weight over time, food intake or health problems between the Bell albinos and Tremper albinos. During the study all the geckos were weighed once a week and there was no significant difference in bodyweight between the two different albino types (p=0.290; see Graph 1). One male however deceased during the course of the study (Tremper albino male 1a), but this was a Tremper albino male and not a ‘weaker’ Bell albino. I have therefore no indications that the statement of a ‘weaker Bell albino morph’ is true. The difference in paternity in this study is remarkable, but whether the difference is caused by the ‘albino type’ remains illusive.

5.3 Copulation duration and paternity

The results show that copulation duration does not affect paternity. The study of Olsson (2001) showed a positive correlation between copulation duration and ejaculate volume, suggesting that a longer copulation would increase the ejaculate volume and thus the change of paternity (Olsson, 2001). In contrast to Olsson (2001) during my study, the males were not able to witness the copulation of his predecessor; both copulations were days or even weeks apart, depending on the gestation period of the first clutch. It is therefore not likely that the ‘second males’ actively invested more time and energy in the copulation to enhance their competitive position. According to literature, there is a positive correlation between males body size and testes size in reptiles (Olsson, 2001; LaDage et al., 2008). There is also a positive correlation found between testes size and ejaculate volume (Iossa et al., 2008). In my study, there was no significant difference found in body weight between the males (see Graph 1) and it is therefore likely that there is no significant difference in testes size and/or ejaculate volume either. No difference in body weight could be found, because the dispersal in body weight is to high and/or the number of males that is used is to low in order to find a difference. The same counts for the copulation duration (see appendix IIb) and no solid statements can be made, due to the low number of observations (N=14). It is likely that if the study is done with more observations, a difference and/or correlation will be found and that copulation does affect paternity. The objective of my study was to use 24 females, but due to problems which are discussed later, the number of observations (=females that contributed to the results) was much lower (N=14).

5.4 Loss of results and other things to consider

At the 21st of May 2008 an accident occurred with the incubator. Due to a technical problem with the heat source, the temperature has risen over night to 47°C. This has proven to be fatal to the eggs and one already hatched juvenile. During this dreadful night, a total of 40 eggs was lost. Of these 40 eggs, 20 eggs counted as ´result-eggs’ (eggs that were laid after the second mating). Because of the loss of these eggs, two females (female #4 and # 8) did not contribute to the results at all, because all their eggs did not hatch. The contribution of six of the in total 14 (contributing) females was damaged; a (big) part of their ‘result-eggs’ died, which had a big influence on all the (quantitative) results of the experiment. Besides the loss of lots of eggs, there are more reasons why not all 24 females contributed to the results.

Female #5 did not show any receptive behaviour when a male tried to mate with her and she even tend to react aggressively. After eight futile attempts, no further efforts were made. This female will be discussed at a later point of this chapter.

Female # 6 did not contributed to the results, because she did not grow fast enough. To prevent health problems, females that weighed less than 40 grams we not allowed to mate (see chapter 2.5). This female did not reach that weight before the end of the breeding season, so no mating attempts took place. The reason for this lack of growth is not known, since the rearing conditions of this individual were identical to those of all the other females.

Female # 11 suffered from fertility problems. She was treated with Paramomicine to recover from an infection with cryptosporidiosis. The influence of the medicine on the reproduction of leopard geckos is not known (Marja Kik, personal communication), but two other females treated with the same medicine did not show any reproductive problems. However, individual metabolically differences could have caused a different reaction to the same medicine (Marja Kik, personal commucation).

Female #18 suffered from weight problems after the first (futile) mating. No further attempts were made because the cause of the problem was not known. No (infectious) decease or parasites could be diagnosed and therefore the reason will remain illusive.

Female #20 did not produce any eggs after the second mating and therefore did not contributed to the results of this experiment.

Female #23 did not produce any eggs at all. Different matings with different males took place, but no eggs were produced. Most likely this female suffered from fertility problems.

Female #24 did not show any receptive behaviour after the first mating. This was a problem because the female could not be ‘loaded’ with the second type of sperm. An explanation for this would be that it was too late in the breeding season for her to show any receptive behaviour.

5.5 Fertility problems

Some females did not produce the number of eggs that were expected or did not produce any eggs at all. Besides that a lot of eggs were lost (in other words “did not hatch”) for reasons unknown. Because the females were quite young (all around one year of age), the first clutch they would produce, were expected to be ‘bad’. It is known that young females have to ‘practice’ in egg production (Vosjoli *et al*., 2005) and the big losses in the first clutches were therefore no surprise. Female virginity was a necessary aspect of the research, so this problem was a necessary evil. It is therefore that eggs were candled to check for fertilization since it was not certain if all the eggs would hatch. However, besides the eggs that were lost due to the failing incubator a substantial part of the eggs did not hatch, especially with females #8 and #15.

Female #8 was diagnosed by Marja Kik (an authority on reptile medicine) with rachites. Since this is a calcium related disease, it is likely to assume that this condition also has a negative effect on the development of the eggs, especially on the calcification proces. The female suffered a short period of time from egg bound and after she laid the (bad) eggs, she was removed from the experiment.

The fecundity problems of female #15 consisted of eggs that looked fine after oviposition. Controlling the eggs for fertilization had a positive outcome; all but one egg were fertilized. Still just four out of the twelve eggs she laid hatched. The reason for this is not known, since the eggs were treated and incubated in the same way as all the other eggs. The caretaking and diet of this female was the same as all the other animals and no health problems were observed in this animal.

Female # 5 did not show any receptive behaviour and every mating attempt was not successful. The reproductive status is believed to have a big influence on receptive behaviour in leopard geckos (Rhen, 2000). It is not likely that this is reason for the shown behaviour, since the mating attempts were scattered over the course of two and a halve months.

5.6 Bell albino males

Two of the four Bell albino males (B2 and B3) that are used in this experiment are not proven to be Bell albinos. In other words: no wild type juveniles hatched as a result of a mating with these males. Males B1 and B4 did produce wild type juveniles and are thus proven to be Bell albinos. Due to chance, the two questionable males mated the most of all males (see the crossing schedule, appendix I) which harms the reliability of the results. Since there are no genetic markers to determine the albino type in leopard geckos (Ron Tremper, personal communication) the only way to determine 'Bell-ness' would be an extra test crossing with a virgin Tremper-albino female. Because the breeding season was on its end when the last eggs hatched, this was not possible anymore. These required test crossings will take place during the next breeding season to be sure which albino type the males really are.

Another possible explanation would be that these males suffer from fertility problems. This is not a probable explanation, because both males B2 and B3 produced fertilized eggs. This contradicts this option and infertility is therefore not a valid explanation. The animals were purchased at a highly recommended leopardgecko breeder (Ray Hine) and it is therefore not likely that both males were accidentally Tremper albinos instead of Bell albinos.

5.7 Recommendations for future research

First the incubator should be more secured than the one is used in this experiment. The use of a professional incubator is recommended. For practical and financial reasons this was not possible for this study, due to the large amount of eggs that was expected and professional incubators tend to be on the small side.

Second the males that are used should be 100% tested for their albino type. There are no known DNA-markers to determine albino-type with leopardgeckos (Ron Tremper, personal communication) so it would be essential to test cross the males before they are used in the experiment. Due to a tight time schedule it was not possible to test cross the albino-males for this study, because the animals were bought as juveniles to make sure that they did not have any experience in mating before.

During this experiment the 'freshness' of the sperm could affect its ability to compete with other sperm. Although the mixed clutches proof that 'old sperm' is still capable to compete with 'fresh sperm' (see chapter 5.1), it would be likely that even more 'mixed clutches' would appear if both types of sperm are equally fresh. If both types of sperm would be equally fresh, it would also rule out the possibility that sperm's 'freshness' affects female cryptic choice (see chapter 2.6), a process that is observed in other species of reptiles (Calsbeek & Sinervo, 2004). In order to rule out the influence of the 'freshness' of sperm on females (cryptic) choice and/or paternity, it would be recommended to let both copulations take place on the same day. It would not be certain however if both types of sperm are actually present in the female and this would be a big disadvantage compared to this study.

The use of DNA-fingerprinting techniques would be more accurate than determine paternity based on phenotype. DNA extraction from leopardgeckos could be relative easy, because of their ability of tail regeneration (Seufer *et al*., 2005; Vosjoli *et al*., 2005). A small piece of the tail could be removed without damaging the animals permanently. However, this method is more invasive and because of animal welfare, the use of this ‘phenotypic’ method is chosen. Bell and Tremper albinos are thought to be compatible every 1 in 400 times (Ron Tremper, personal communication), but this information was not known before the start of the experiment and no (scientific) proof of this (very rare) compatibility of the different albino types could be found.

5.8 Applications for leopard gecko breeding

The set up for this experiment is not a natural one; the females were not able to choose their own mate and the animals just mated once. Leopardgeckos tend to mate more than once before oviposition (LaDage *et al*., 2008) and it is likely that not mating order has an influence on paternity, but that the number of copulations does have an affect on paternity. Olsson (2001) showed that males of the agamid lizard *Ctenophorus fordi* copulate longer and deposit more sperm during copulation as a tool in sperm competition (Olsson, 2001). It is likely that also an increase in the number of copulations has its affect on paternity; if one male is able to place more spermatozoa inside a female than another male, the first male stands a bigger chance to sire the juveniles. This could be of use for (professional) reptile breeders. Many females are used for breeding purposes for more that one year. During the females life span, she usually copulates with many different males in order to create different morphs of high quality offspring (Eelco Schut, personal communication; personal experience). If mating order does not affect paternity, breeders could decide to let the female mate more often with the male they would like to get juveniles from.

Acknowledgements

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Literature

Baird T.A., 2008. A growth cost of experimentally induced conspicuous coloration in first-year collared lizard males, *Behavioral Ecology* 19 (3): 589-593

Birkhead T.R., Møller A.P., 1993. Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals, *Biological Journal of the Linnean Society* 50 (4): 295-311

Birkhead T.R., Pizzari T., 2002. Post-copulatory sexual selection, *Nature Reviews Genetics* 3 (4): 262–273

Bragg W.K., Fawcett J.D., Bragg T.B., Viets B.E., 2000. Nest-side selection in eublepharid gecko secies with temperature-dependent sex determination and one with genotypic sex determination, *Biological Journal of the Linnean Society* 69: 319-322

Calsbeek R., Sinervo B., 2004. Within-clutch variation in offspring sex determined by differences in sire body size: cryptic mate choice in the wild, *Journal of Evolutionary Biology* 17 (2): 464–470

Cook, P. A., Wedell, N. 1999. Non-fertile sperm delay female remating. *Nature* 397 (6719): 486

Cooper W.E., Vitt L.J., 1997. Maximizing male reproductive success in the broad-headed skink (*Eumeces laticeps*): Preliminary evidence for mate guarding, size-assortative pairing, and opportunistic extra-pair mating**,** *Amphibia Reptilia*, 18 (1): 59-73.

Cox R.M., Skelly S.L., John-Alder H.B., 2003. A comparative test of adaptive hypotheses for sexual size dimorphism in lizards, *Evolution* 57 (7): 1653-1669

Evans J.P., Simmons L.W., 2007. The genetic basis of traits regulating sperm competition and polyandry: can selection favour the evolution of good- and sexy sperm?, *Genetica* 134 (1): 5–19

Flores D., Tousignant A., Crews D., 1994. Incubation Temperature affects the behavior of adult Leopard geckos (*Eublepharis macularius*), *Physiology & Behavior* 55(6): 1067-1072

Foellmer, M.W., 2008. Broken genitals function as mating plugs and affect sex ratios in the orb-web spider *Agriope aurantia*, *Evolutionary Ecology Research* 10 (3): 449-462

Gamble T. Aherns J.L., Card V., 2006. Tyrosinase Activity in the Skin of Three Strains of Albino Gecko (*Eublepharis macularius*), *Gekko* 5 (1): 39-44

Girling J.E., Cree A., Guillette L.J., 1997. Oviductal Structure in a Viviparous New Zealand Gecko, *Holodactylus maculatus*, *Journal of Morphology* 234: 51–68

Harris W.E., 2008. Spermatophore deposition behaviour in an explosive breeder, the Small-mouthed salamander, *Ambystom texanum*, *Herpetologica*, 64 (2): 149–155

Hosken D.J., Taylor M.L., Hoyle K., Higgins S., Wedell N., 2008. Attractive males have greater success in sperm competition, *Current Biology* 18 (13): R553-R554

Hunter, F.M., Petrie, M., Otronen, M., Birkhead, T., Møller, A.P., 1993. Why do females copulate repeatedly with one male?, *Trends in Ecology and Evolution* 8 (1): 21-26

Iossa G., Soulsbury C.D., Baker, P.J. Harris, S., 2008. Sperm competition and the evolution of testes size in terrestrial mammalian carnivores, *Functional Ecology* 22 (4): 655–662

Jennions M.D. , Petrie M., 2000. Why do females mate multiply? A review of the genetic benefits, *Biological Reviews* 75 (1): 21-64

Johnston E.E., Rand M.S., Zweifel S.G., 2006. Detection of multiple paternity and sperm storage in a captive colony of the central Asian tortoise, *Testudo horsfieldii*, *Canadian Journal of Zoology* 84 (1): 520-526

Johnston G., Bouskila A., 2007. Sexual dimorphism and ecology of the gecko, *Ptyodactylus guttatus*, *Journal of Herpetology* 41 (3): 506-513

McCoy J.K., Baird T.A., Fox, S.F., 2003. Sexual selection, social behaviour and the environmental potential for polygyny, *Lizard Social Behaviour*, The Jonghs Hopkins University Press, London

Moreira P.L., Birkhead T.R. , 2003. Copulatory plugs in the Iberian Rock Lizard do not prevent insemination by rival males, *Functional Ecology* 17 (6): 796-802

Moreira P.L., Birkhead T.R., 2004. Copulatory plug displacement and prolonged copulation in the Iberian rock lizard (*Lacerta monticola*), *Behavioral Ecology and Sociobiology* 56 (3): 290-297

Moreira P.L., Nunes V.L., Martín J., Paulo O.S., 2007. Copulatory plugs do not assure high first male fertilisation success: Sperm displacement in a lizard, *Behavioral Ecology and Sociobiology* 62 (2): 281-288

Murphy-Walker S., Haley S.R., 1996. Functional sperm storage duration in female *Hemidactylus frenatus* (family Gekkonidae), *Herpetologica* 52 (3): 365-373

LaDage L.D., Ferkin M.H., 2008. Do conspecific cues affect follicular development in the female leopard gecko (*Eublepharis macularius*)?, *Behaviour* 145 (8): 1027-1039

LaDage L.D., Gutzke W.H.N., Simmons II R.A., Ferkin M.H., 2008. Multiple mating increases fecundity, fertility and relative clutch mass in the female Leopard gecko (*Eublepharis macularius*), *Ethology* 114 (5): 512–520

Lewis S.M., Austad S.N.,1994. Sexual selection in flour beetles: the relationship between sperm precedence and male olfactory attractiveness, *Behavioural Ecology* 5 (2): 219–224

Olsson M., Gullberg A., Tegelstrom H., 1994. Sperm competition in the Sand Lizard, *Lacerta agilis*, *Animal Behaviour* 48 (1): 193-200

Olsson M., Madsen T., 1995. Female choice on male quantitative traits in lizards- why is it so rare?, *Behavioural Ecology and Sociobiology* 36 (3): 179 184

Olsson, M., 2001. ‘Voyeurism’ prolongs copulation in the dragon lizard *Ctenophorus fordi, Behavioral Ecology and Sociobiology* 50 (4): 378-381

Olsson M., Healey M., Wapstra E., Schwartz T., Lebas N., Uller T., 2007. Mating system variation and morph fluctuations in a polymorphic lizard, *Molecular Ecology* 16 (24): 5307–5315

Olsson M., Wapstra E., Healey M., Schwartz T., Uller T., 2008. Selection on space use in a polymorphic lizard, *Evolutionary Ecology Research* 10 (4): 621–627

Oppliger A., Degen L., Bouteiller-Reuter C., John-Alder H.B., 2007. Promiscuity and high level of multiple paternity in common wall lizards (*Podarcis muralis*): data from microsatellite markers, *Amphibia-Reptilia* 28 (2): 301-303

Pearse D.E., Janzenà F.J., Avise J.C., 2001. Genetic markers substantiate long-term storage and utilization of sperm by female painted turtles, *Heredity* 86 (3): 378-384

Price T.A.R., Wedell N., 2008. Selfish genetic elements and sexual selection: their impact on male fertility, *Genetica* 134 (1): 99-111

Pizzari T., Birkhead T.R., 2000. Female feral fowl eject sperm of subdominant males, *Nature* 405 (6788): 787-789

Pizzari T, Cornwallis C.K., Løvlie H., Jakobsson S., Birkhead T.R., 2003. Sophisticated sperm allocation in male fowl, *Nature* 426 (6962): 70-74

Rhen T., Sakata J.T., Zeller M., Crews D., 2000. Sex steroid levels across the reproductive cycle of female leopard geckos, *Eublepharis macularius*, from different incubation temperatures, *General Comparative Endocrinology* 118: 322-331

Rosengrave P., Gemmell N.J., Metcalf V., McBride K., Montgomerie R., 2008. A mechanism for cryptic female choice in chinook salmon, *Behavioral Ecology* 19 (6): 1179-1185

Rösler H, *Vermehrung von Geckos*, 2005. Herpeton, Offenbach

Schulte-Hostedde A.I., Montgomerie R., 2006. Intraspecific variation in ejaculate traits of the northern watersnake (*Nerodia sipedon*), *Journal of Zoology* 270 (1): 147–152

Seufer H., Kaverkin Y., Kirschner A., *The Eyelash Geckos, care, breeding and natural history*, 2005. Kirschner & Seufer Verlag, Karlsruhe

Schumacher R., 2002. *Halsbandleguane, Lebensweise, Pflege, Zucht*, Herpeton, Offenbach

Stuart-Smith J., Swain R., Wapstra E., 2007. The role of body size in competition and mate choice in an agamid with female-biased size dimorphism, *Behaviour* 144: 1087-1102

Uller T., Olsson M., 2008. Multiple paternity in reptiles: patterns and processes, *Molecular Ecology* 17 (11): 2566–2580

Viets B.E., Tousighant A., Ewert M.A., Nelson C.E., Crews D., 1993. Temperature-dependent sex determination in the leopard gecko, *Eublepharis macularius*, *The journal of experimental zoology* 265: 679-683

Vosjoli de P., Tremper R., Klingenberg R., DVM, *The herpetoculture of Leopard Geckos, twenty-seven generations of Living Art*, 2005. Advanced Visions, Sterling

Yuval B, 2006. Mating systems of blood-feeding flies, *Annual Review of Entomology* 51:413–440

Zamudio K.R., Sinervo E., 2000. Polygyny, mate-guarding, and posthumous fertilization as alternative male mating strategies, *Proceedings of the National Academy of Sciences* 97 (26): 14427–14432.

Figures 1, 4, 6, 8, 9, 11, 12 and 22, photo by Yuri Stevens
Figure 5: Tremper albino leopardgecko, photo by Eugène Bruins, curator Artis
Figure 7: Rainwater albino leopardgecko, photo by Jakub Dolezel, website: http://www.leopard-gecko-cz.com, visited at 04-06-2008
Figures 13 – 21 (photos of the embryos and foetuses) are made by Lorenzo Fanchi

Appendix

Appendix I Crossing diagram

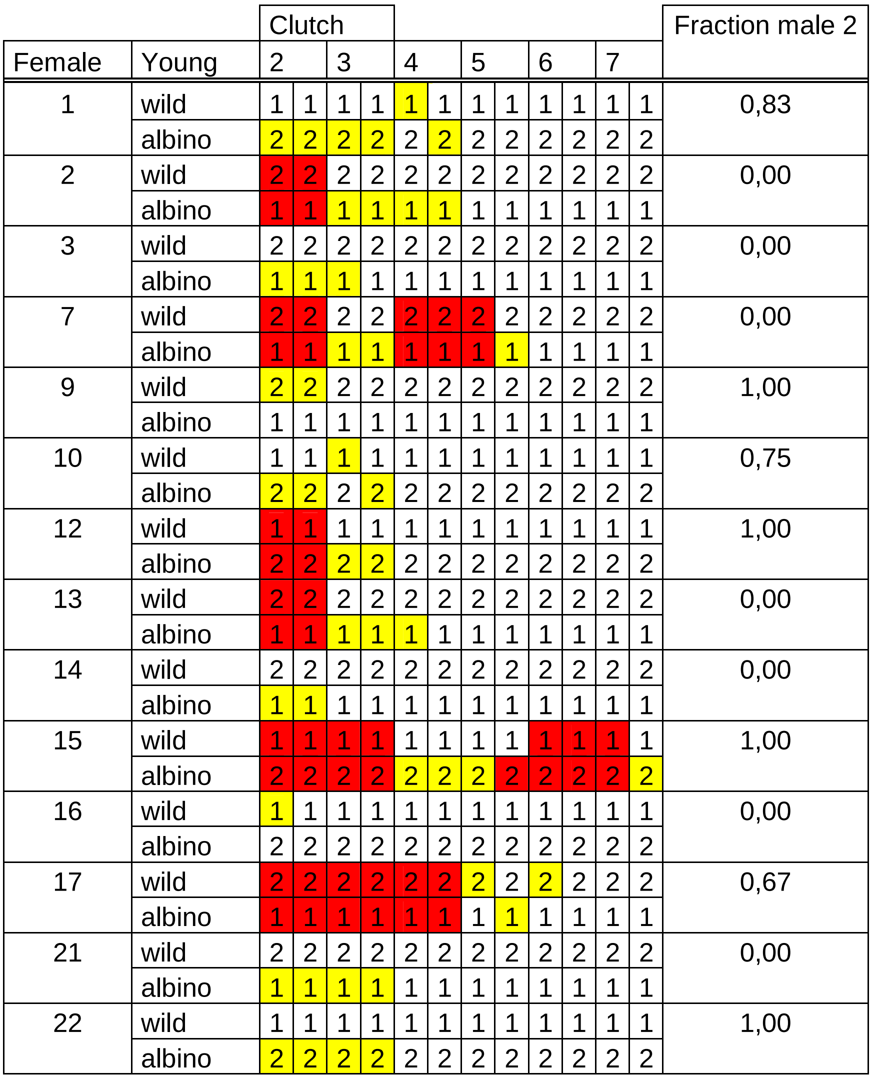
The way to read table 2 is as follows: female 21 (highlighted in yellow in table a) is first mated with male T1 (Tremper albino 1) and after she laid the first (fertile) clutch, she is mated with male B1 (Bell albino 1). Female 24 is first mated with male B1 and after the first (fertile) clutch, she is mated with male T1. The reciprocal crossings are made to control for the possibility that one male possesses stronger or more viable sperm than the other male.

Female no.	male couples	Female no.
21	T1-B1	24
9	T4-B1	1
2	T3-B2	6
23	T1-B4	19
7	T2-B3	12
18	T1-B3	8
17	T2-B1	16
14	T1-B2	5
13	T4-B3	15
22	T2-B2	20
3	T3-B3	4
11	T4-B4	10
	T2-B4	
	T3-B4	
	T4-B2	
	T3-B1	

Table 2: Crossing diagram, the bottom four male couples are not used due to a surplus of male–male combinations.

Appendix IIa Mating order

Table 3 shows the results of the experiment. The column ‘Female’ shows which female the results belong to. ‘Young’ describes the phenotype of the juveniles; wildtype (wild) or albino. ‘Clutch’ refers to the clutch number. The digits ‘1’ and ‘2’ represents the mating of which the juvenile is a product of (according to the phenotype). For example: Both juveniles hatched out of the second clutch of female 1 were a product of the second mating, while one of the juveniles of the fourth clutch of the same female is a product of the first mating. ‘Fraction male 2’ is the fraction is tested. The red boxes represent eggs that didn’t hatch and are thus not considered as a result, only the yellow boxes count as results.



		Clutch												Fraction male 2
Female	Young	2		3		4		5		6		7		
1	wild	1	1	1	1	1	1	1	1	1	1	1	1	0,83
	albino	2	2	2	2	2	2	2	2	2	2	2	2	
2	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
3	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
7	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
9	wild	2	2	2	2	2	2	2	2	2	2	2	2	1,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
10	wild	1	1	1	1	1	1	1	1	1	1	1	1	0,75
	albino	2	2	2	2	2	2	2	2	2	2	2	2	
12	wild	1	1	1	1	1	1	1	1	1	1	1	1	1,00
	albino	2	2	2	2	2	2	2	2	2	2	2	2	
13	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
14	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
15	wild	1	1	1	1	1	1	1	1	1	1	1	1	1,00
	albino	2	2	2	2	2	2	2	2	2	2	2	2	
16	wild	1	1	1	1	1	1	1	1	1	1	1	1	0,00
	albino	2	2	2	2	2	2	2	2	2	2	2	2	
17	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,67
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
21	wild	2	2	2	2	2	2	2	2	2	2	2	2	0,00
	albino	1	1	1	1	1	1	1	1	1	1	1	1	
22	wild	1	1	1	1	1	1	1	1	1	1	1	1	1,00
	albino	2	2	2	2	2	2	2	2	2	2	2	2	

Table 3: The table shows the complete data

Appendix IIb Bell vs. Tremper and copulation duration

Table 4 shows which juveniles were produced by a Tremper albino male and which by a Bell albino male. “Fraction Bell” is the fraction of juveniles that is sired by a Bell-albino male. The cells containing ‘Tremper’ of ‘Bell’ correspond to the yellow coloured cells in table 3 (see: appendix IIa).

Female#							Fraction Bell
1	Tremper	Tremper	Tremper	Tremper	Bell	Tremper	0,17
2	Tremper	Tremper	Tremper	Tremper			0,00
3	Tremper	Tremper	Tremper				0,00
7	Tremper	Tremper	Tremper				0,00
9	Bell	Bell					1,00
10	Tremper	Tremper	Bell	Tremper			0,25
12	Tremper	Tremper					0,00
13	Tremper	Tremper	Tremper				0,00
14	Tremper	Tremper					0,00
15	Tremper	Tremper	Tremper	Tremper			0,00
16	Bell						1,00
17	Bell	Tremper	Bell				0,67
21	Tremper	Tremper	Tremper	Tremper			0,00
22	Tremper	Tremper	Tremper	Tremper			0,00

Table 4: The table shows whether the Tremper of the Bell male sired the juveniles.

Table 5 shows the copulation duration data. The first column shows the number of the female. The second shows the fraction of juveniles that was a result from the second mating. If a value > 0.5, the second copulation is considered to be the “winner” (last column). If a value < 0.5, the first copulation is considered to be the “winner” (last column). The two columns show “cop1” and “cop2” show the duration of copulation 1 resp. copulation 2.

female	fraction 2	cop 1	cop 2	"winner"
1	0,83	19,15	53,78	2
2	0,00	31,83	33,16	1
3	0,00	37,2	44,62	1
7	0,00	34,04	25,42	1
9	1,00	51,52	12,97	2
10	0,75	35,32	33,7	2
12	1,00	111,16	87,77	2
13	0,00	47,56	30,83	1
14	0,00	81,79	70,45	1
15	1,00	25,65	106,36	2
16	0,00	28,02	46,83	1
17	0,67	28,99	31,92	2
21	0,00	81,02	51,44	1
22	1,00	61,52	30,79	2

Table 5: The table shows if the biggest fraction of juveniles is a product of the first or the second copulation.

Appendix IIc Copulation duration: Bell vs. Tremper and body weight

Table 6 show the albino-type and copulation duration in seconds. Only the copulation that resulted in hatched eggs are counted for as results.

Albino-type	Duration	Albino-type	Duration
Tremper	81,02	Bell	19,15
Tremper	81,79	Bell	31,92
Tremper	34,04	Bell	28,02
Tremper	28,99	Bell	12,97
Tremper	87,77	Bell	33,16
Tremper	46,83	Bell	61,52
Tremper	30,79	Bell	51,44
Tremper	31,83	Bell	70,45
Tremper	37,2	Bell	111,16
Tremper	47,56	Bell	25,65
Tremper	106,36	Bell	25,42
Tremper	51,52	Bell	30,83
Tremper	53,78	Bell	44,62
Tremper	33,7	Bell	35,32

Table 6: Albino-type and copulation duration measured in seconds.

Tabel 7 shows the weight of each male over time. T= Tremper, B= Bell. Male T1a deceased after 6th of May and male T1 was added to the study in his place.

Datum:	T1a	T1	T2	T3	T4	B1	B2	B3	B4
12-03-08	50		44	80	67	48	51	54	58,0
18-03-08	47,7		42,2	79,9	68,4	48,4	52	53,4	54,6
25-03-08	44,3		43,6	81,9	67,3	47,3	-	53,9	57,1
1-04-08	45,6		49,8	81,3	67,3	47,6	57	56,2	57,6
8-04-08	46,5		50,5	79,6	68,7	50,2	56,8	54,6	58,6
15-04-08	48,7		50,7	81,1	67,1	50,4	57,7	54,4	58,3
22-04-08	48		49,8	80,3	65,9	49,8	56,7	53,1	59,9
29-04-08	48,8		52,1	80,3	64,5	49,6	59,1	54,6	61,1
6-05-08	49,4		51,7	78,8	66,8	49,3	58,5	54,9	57,8
13-05-08		61,4	51,9	80,3	65,5	51,3	60,4	55,4	60,0
20-05-08		61,9	55,5	81,4	68,2	52,6	62,8	58,2	61,4
28-05-08		58,7	55,8	80,4	67,3	52,4	63,6	58,5	62,3
3-06-08		58,2	57,1	80,6	66,7	53	65,6	58,9	63,2
10-06-08		60,1	57,4	84,4	70,4	53,9	66,1	57,9	63,9
17-06-08		59,7	58,4	83,7	68,9	56,2	68,2	59,7	65,2
24-06-08		60,9	59,2	83,1	69,2	56,2	68,1	63	65
1-07-08									
8-07-08		61,9	61,5	85,8	72,9	58,7	68,7	64,7	69,8
15-07-08		60,4	63,4	84,1	71	59,8	71,9	65,6	69,7
22-07-08		62	63,8	86,6	74,3	59,1	73,1	66,5	71,1
29-07-08		63,2	63,7	87,9	72,6	58,1	74,3	68,3	71,2
5-08-08		63,8	65,3	87,8	75,5	61,3	75,9	69,9	71,1
12-08-08		62,8	65,7	90,2	72,8	59,6	78,6	72,3	73,4
19-08-08		62,5	66,9	89,1	73	58,6	79,1	70,3	72,4

Table 7: The weight of every male over time (in grams).

Appendix IIIa Output of SPSS

T-Test Fraction second mating

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Fraction2	14	,4464	,47313	,12645

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
Fraction2	-,424	13	,679	-,05357	-,3268	,2196

T-Test Fraction Bell

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
FractionBell	14	,2207	,37808	,10105

One-Sample Test

	Test Value = 0.5					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
FractionBell	-2,764	13	,016	-,27929	-,4976	-,0610

Copulation Duration

Group Statistics

	albinotype	N	Mean	Std. Deviation	Std. Error Mean
duration	1	14	53,7986	25,16301	6,72510
	2	14	41,5450	25,59790	6,84133

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
duration	Equal variances assumed	,065	,801	1,277	26	,213	12,25357	9,59326	-7,46566	31,9728
	Equal variances not assumed			1,277	25,992	,213	12,25357	9,59326	-7,46595	31,9731

Appendix IIIb Output of SPSS

Copulation duration and paternity

Group Statistics

	father	N	Mean	Std. Deviation	Std. Error Mean
Duration	0	14	45,4329	24,01863	6,41925
	1	14	49,9107	27,95690	7,47180

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
Duration	Equal variances assumed	1,015	,323	-,455	26	,653	-4,4779	9,8506	-24,7261	15,770
	Equal variances not assumed			-,455	25,423	,653	-4,4779	9,8506	-24,7485	15,793

Difference in body weight over time: Tremper-albino males and Bell-albino males

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	567849,386	1	567849,386	232,212	,000
VAR00006	3627,219	1	3627,219	1,483	,290
Error	9781,559	4	2445,390		