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Antimicrobial defense mechanisms in reptile eggs

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2 BACKGROUND

2.1 THE AMNIOTE EGG

The colonization of land by vertebrate animals started in the late Devonian, more than 380 million years ago. The transition to a terrestrial lifestyle happened gradually as it required multiple adaptations. One striking example is that of the fossil species *Tiktaalik.* This species is well known among biologists because it possessed features that are intermediate between fish and early tetrapod lineages, indicating that its body plan closely resembles the ancestors of a new major vertebrate clade, the Tetrapoda (Futuyma & Kirkpatrick, 2017). Although species like *Tiktaalik* and their relatives were able to breath air and support their body on land, they were not fully terrestrial. They deposited their eggs in water and their hatchlings lived aquatically until completion of development. As such, freshwater bodies remained fundamental for the survival of the early Tetrapoda and their lifecycle can be compared to that of extant amphibians today (Blackburn & Stewart, 2021).

Although multiple features evolved that enhanced adult life on land, it was not until 312 million years ago that an early tetrapod lineage was able to fully decouple its lifecycle from the water. This event was the result of the evolution of the amniote egg and gave rise to an entirely new clade, the so-called Amniota. From this point onwards, freshwater bodies were no longer a necessity for eggs to develop and early amniotes were able to further colonize the land. More than 60 million years after *Tiktaalik* started to venture itself above the water surface, vertebrates started to flourish in a vast range of terrestrial habitats. This was the start of a massive radiation and eventually resulted in some of the most successful clades that ever existed. Extant mammals, reptiles and birds are the living remnants of this success and represent around 43% of all the vertebrates that exist today (Futuyma & Kirkpatrick, 2017).

What differentiates the amniote egg from the more primitive vertebrate egg design, is the addition of extraembryonic membranes and a shell. Research suggest that it is highly unlikely that the amniote egg evolved more than once during vertebrate history (Sander, 2012).This means that early amniotes already possessed this feature because the Synapsids (i.e. mammals) and the Reptilia diverged relatively soon after the origin of the amniote clade. The latter group will be the focus of this research because the egg itself is has been lost in mammals, more derived than monotremes.

There is a vast amount of diversity in the eggs found within the Reptilia. This is not very surprising given the fact that the eggs we find today are the result of more than 300 million years of evolutionary processes (Blackburn & Stewart, 2021). In addition, fossils of the first amniote eggs are very hard to come by because shell-less and soft-shelled eggs are unlikely to preserve by fossilization (Sander, 2012). Recently, a very unique, soft-shelled egg was discovered and shows similarities to eggs of extant lizards and snakes. The fossil is believed to belong to a large marine reptile that lived roughly 68 million years ago and proves once again the immense diversity encompassed by the amniote egg (Legendre et al., 2020). Nevertheless, many features are highly conserved and some generalizations can still be made. An overview is shown in Figure 1[1].

[1] Note that the egg, as presented in Figure 1, is merely a sketch of the different layers and does not represent the ancestral state, nor does it represent the egg of a particular species.

In the ancestral mode, as in amphibians today, egg laying is characterized by the deposition of large clutches by the female. The male fertilises them immediately after they are laid in the water. They are encapsuled in a protective jelly layer which rapidly increases in size the moment it comes in touch with water. The developing embryo can easily exchange gases and nitrogenous waste with its aquatic environment, simply by diffusion. Most of the species have at least one larval stage wherein the larvae swim within the water and provide themselves with the necessary food sources (Vitt & Caldwell, 2009).

In the Reptilian clade however, there are some important differences. In contrast with their water dependent ancestors, amniote species do not have a larval stage in their lifecycle. Hatchlings represent their adult body plan and are relatively large compared to their anamniote counterparts. In addition, amniote eggs develop on dry land and free exchange with the environment would cause lethal damage due to water loss. This means that they cannot rely on diffusion to fulfil the nutritional needs of the embryo. As a result, the amniote egg has to contain all the energy and nutrients needed for successful development, before it is laid (Vitt & Caldwell, 2009).

This function is provided by the yolk and is already present in the female gamete, prior to ovulation. During the period of reproductive activity the ova grow immensely due to the storage of yolk causing a mature ovum to be 10-100 times its original size. It consists mainly of lipids and proteins and is typically yellow in colour. The yolk is the single food source to sustain the embryo through the prolonged egg-stage up until hatching (G. C. . Packard & Packard, 2009). As a result, the amniote egg is characterized by a very large yolk.

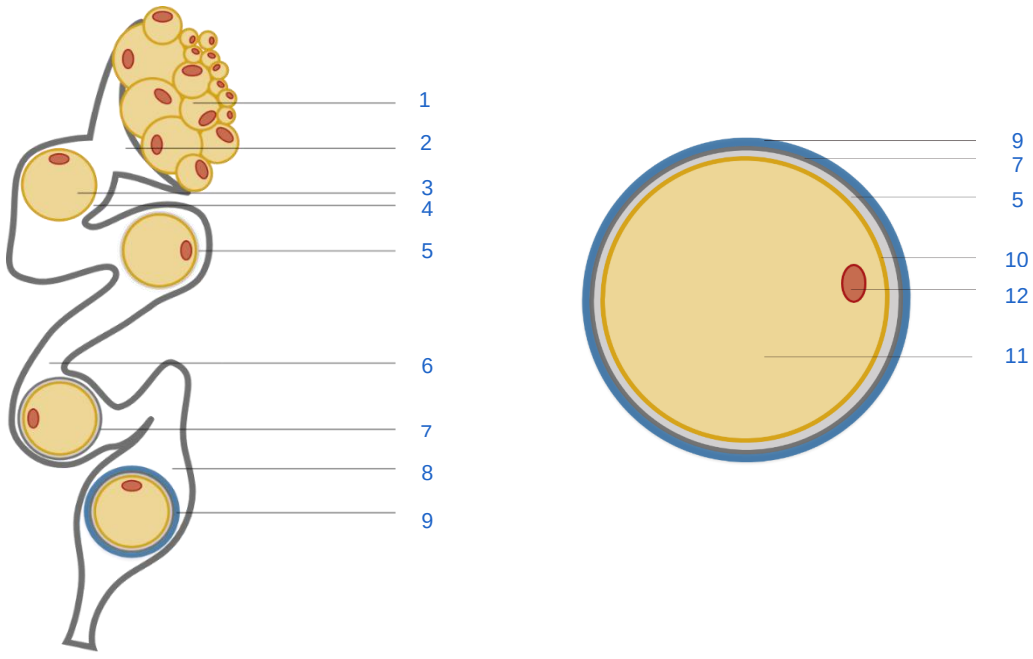

Figure 1: Generalised scheme of amniote egg formation in the reproductive tract (left) and close up (right).

1. Unmature ova in ovary, 2. Infundibulum, 3. Mature ovum after ovulation, 4. Potential fertilization, 5. Albumen, 6. Isthmus, 7. Shell membrane, 8. Uterus, 9. Eggshell, 10. Vitelline membrane, 11. Yolk, 12. Germ disc

Potential fertilisation happens soon after ovulation (Vitt & Caldwell, 2009). The DNA is present in one spot near the surface in what is called the germ disc. In all birds and some non-avian reptiles, the large ovum gets surrounded in albumen while traveling further through the reproductive tract. Albumen is a transparent, jelly layer consisting mainly of proteins, glycoproteins and water (Mann, 2007). It serves both as a physical and chemical buffer, protecting the embryo from damage due to microbes, mechanical forces and water loss (Deeming & Ferguson, 2003).

When arrived in the uterus, the egg is packed in a shell. It consists of one or two organic membranes and, depending on the species, a calcium-rich outer layer. The shell serves as a barrier between the inside of the egg and the outside world. As a result, the embryo is able to develop in its own microenvironment protected from diverse kinds of potential damage (Deeming & Ferguson, 2003).

As a result, the amniote egg is almost completely able to exist independently, aside from maternal incubation in most birds and some reptiles. After oviposition, the egg develops in a nest or nest chamber, in or above the ground (Refsnider, 2016).

Amniote eggs are characterized by the presence of foetal membranes (Futuyma & Kirkpatrick, 2017). During embryogenesis, the amnion, chorion and allantois grow from embryonic tissue. They are adaptations and help the embryo to cope up with the harsh conditions faced on land. An overview is given in Figure 2. The innermost membrane is called the amnion. It is filled with fluid and forms the amniotic cavity in which the embryo develops. This membrane is thought to protect against fluid loss and to prevent tissue adhesion (Deeming & Ferguson, 2003).

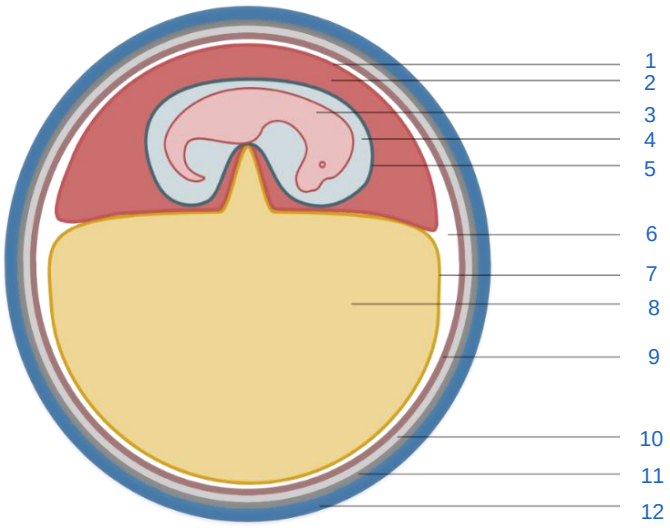

Figure 2: Generalised scheme of a developing amniote embryo with foetal membranes.

1. Allantois, 2. Waste products, 3. Embryo, 4. Amniotic cavity, 5. Amnion, 6. Chorionic cavity, 7. Yolk sac, 8. Yolk, 9. Chorion, 10. Albumen, 11. Shell membrane, 12. Eggshell

Early in development, pore-like structures in the shell allow the embryo to exchange gases with the surrounding environment by diffusion. However, as the embryo grows, so does its need for oxygen (Deeming & Ferguson, 2003). The allantois is a sac-like membrane that will become highly vascularized as growth progresses. The highly branched veins enlarge the surface area through which diffusion of gases can occur. Oxygen is more easily taken up while carbon dioxide can leave the body before suffocating the foetus. In addition, the allantois filters the blood by serving as a temporary kidney.

Nitrogenous waste cannot be disposed of though the pores and high amounts in the body can cause lethal damage. In order to prevent this from happening, the nitrogen is stored inside the allantois where it does not interfere with the metabolism of the foetus. The third membrane characterizing the egg is the chorion. This structure encloses the embryo and the rest of the embryonic system. This layer is also filled with fluid and therefore serving as a shock absorber during development. In a later stage, it fuses with the allantois and together they advance oxygen supply. (Blackburn & Flemming, 2009)

2.2 AMNIOTE EGG DEFENCE MECHANISMS

The large yolk is a very nutritious food source, not only for the growing embryo, but for almost all life forms on Earth. The relatively thick eggshell forms the first layer of physical protection. It is probably selected for, during evolutionary history, as it effectively prevents many macropredators from reaching the coveted content of the egg (G. C. . Packard & Packard, 2009). Predation has been recognized as the major cause of egg mortality (DeSana et al., 2020) In a less traditional sense, predation could also be considered as the consumption of egg components by heterotrophic bacteria (Board and Hornsey 1978). Indeed, the intricate structure of eggshells and albumen likely evolved from the ancestral amniotic egg at least partly as a result of intense predation by microbes (G. C. Packard & Packard, 1980).

Numerous studies over the past 30 years have examined the susceptibility of eggs to contamination by bacteria. However, most have focused on domestic avian species (primarily chickens), with the goal of improving egg hygiene and thereby reducing loss due to contamination (Tranter & Board, 1982; Hincke et al., 2011). Broadly speaking, this research has shown that microbial infection affects reptile and bird eggs in the wild (e.g., Wyneken et al., 1988; Pinowski et al. 1994), that certain environmental conditions promote egg contamination, and that vertebrates have evolved numerous behavioural, chemical and physiological adaptations to combat infection (reviewed in D'Alba & Shawkey, 2015).

It is much less clear how reptiles are able to deal with predation by microorganisms. Developing embryos do not have an immune system yet and unlike mammals they cannot rely on antibodies rendered by the mother. In addition, microorganisms are known to be very abundant in nesting environments and infection can cause the egg to fail.

To understand exactly how embryos are protected from destruction by pathogens, microbial defence mechanisms in bird eggs will be described first.

2.2.1 Bird egg structure

Birds form a relatively young clade from an evolutionary point of view. They are characterized by some key features that distinguish them from their reptilian ancestors. As a result, birds are often seen as more derived when compared to non-avian reptiles existing today. The avian clade is very successful with over 10000 living species. They are exotherm with a body temperature around 40°C. This allows them to incubate their eggs and reproduce at latitudes

that are even higher than the polar circle. In addition, the avian egg is often described as one of nature's most perfect designs (Birkhead M. , 2018; Birkhead T. , 2016). It exists in a wide range of sizes, shapes, colours and colour patterns. A scheme is shown in Figure 3.

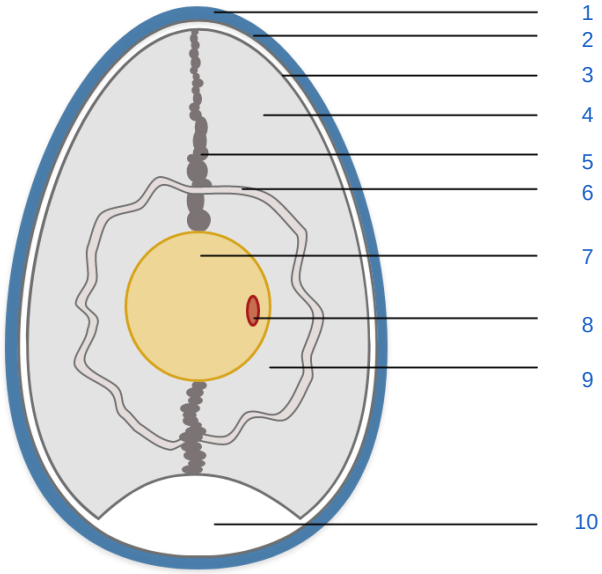

Figure 3: Schematic representation of a bird egg

1. Eggshell, 2. Outer shell membrane, 3. Inner shell membrane, 4. Thin albumen, 5. Chalaza, 6. Intermediate layer, 7. Yolk, 8. Germ disc, 9. Thick albumen, 10. Air sac

2.2.1.1 The shell and cuticle

Bird eggs differentiate themselves in almost every level of organisation. The most remarkable feature is their highly calcified shell. They are always hard and brittle while the shell of non-avian reptile species is often parchment-like and pliable. This implies a highly organised composition of calcium crystals which are formed in the uterus. The crystals grow outwards, starting from nuclei attached to the shell membrane. As they grow larger, they merge with one another and eventually form a closed layer. In between the originally stacked crystals, tiny spaces remain and form pores which go all the way through the shell. These pores are necessary for the embryo to breathe by exchanging gases with its outer environment (Figure 5, left).

Microorganisms can enter the egg in two different manners. The first possible way is the infection by pathogens while the egg is being formed inside the reproductive tract of the mother. The resulting contamination is often called 'vertical transmission'. Depending on the location of the infected organ, the microbe can skip the shell, shell membranes and sometimes even the albumen, resulting in the direct infection of the egg part that is formed by that respective organ (Gantois et al., 2009).

The other way is called horizontal transmission and occurs when microbes infect the egg after it is formed and/or laid. This route implies the penetration of the shell and shell membranes. This happens most easily through the pores and bacteria do not necessarily harm the shell's structure. To prevent this from happening most eggs contain an additional water-repellent layer on top of the shell (B. Y. R. G. Board & Fuller, 1973). This structure is called the cuticle and

renders additional protection by effectively blocking the entrance of the pores and negatively affects attachment of bacteria onto the shell (D'Alba et al., 2016, 2017), (Figure 5, left B).

However, when the egg gets wet, microbes are thought to overcome this barrier quite easily. Osmotic pressure, can suck the microbes through the pores and especially when the egg is drying.(R. G. Board & Fuller, 1994).

2.2.1.2 Shell membranes

Once passed through the calcified layer, the microbes have to overcome the two shell membranes. They consist of a meshwork of interlaced fibres. The main components are collagen (10%) and other proteins and glycoproteins that contain lysine-derived cross-links (70-75%) (Hincke et al., 2012).

The innermost membrane is the thinnest but the most effective against bacterial penetration, both physical and chemical. The dense network prevents the formation of pores while the presence of lysozyme causes gram-positive bacteria to lyse (Baker, 1974).

Bacteria are thought to enter by wiggling their way through the fibrous network while infiltrating hyphae of moulds are nearly unhindered by the membranes (Baker, 1974).

2.2.1.3 Albumen

The anti-microbial properties of chicken albumen are well understood. A multitude of factors make it hard for microbes to stay alive and cross the thick, jelly-layer.

The most obvious protection is rendered by its low nutritious value to microbes. In his book, expert Tim Birkhead describes such a passage as the human equivalent of walking through the Atacama Desert: there is nothing to keep them alive (Birkhead, 2016). In addition; the albumen is alkaline (pH 9-10), which further negatively impacts microbial growth (Baker, 1974; Board & Fuller, 1973; Birkhead, 2016).

The egg white contains 11% of the mass consist of proteins, whereof some negatively influence the microbial metabolism (B. Y. R. G. Board & Fuller, 1973). Up to 148 proteins have been detected in the albumen but not all of them perform an antimicrobial function (Mann, 2007) and new enzymes are probably still to be discovered (Kaweewong et al., 2013). They generally have a very low molecular weight and are therefore often referred to as peptides (Mann, 2007). The most abundant ones will be described here and are listed in Table 1.

Ovalbumin is the most abundant peptide and therefore one of the first to be discovered in chicken albumen. It is described as a storage protein (*SERPINB14 - Ovalbumin - Gallus Gallus (Chicken) - SERPINB14 Gene & Protein*, n.d.) and does not act as an antimicrobial protein as a whole. However, after cleavage, some derivates have proven to be successful in inhibiting gram- positive and -negative bacteria as well as fungi (Tan & Yokoyama, 2020).

Ovotransferrin is a protein that inhibits bacterial growth. It contains two domains that can each bind an iron molecule. This way, the iron is made unavailable for microbial use within the albumen. By creating an iron-deprived environment, ovotransferrin performs a bacteriostatic function (Wellman-Labadie et al., 2007).

Ovomucoid inhibits trypsin (*Ovomucoid Precursor - Gallus Gallus (Chicken)*, n.d.). Trypsin belongs a group of enzymes that cleaves specific peptide bonds in proteins, breaking them down into smaller units. Bacteria use this enzyme to facilitate digestion (Pilon et al., 2017). By

inhibiting this process, ovomucoid acts as bacteriostatic protein, making it more difficult for microbes to survive in the already nutrient-scarce albumen.

Lysozyme is an enzyme found in many eukaryotic cell secretions and functions mostly as a self-defence mechanism. It is divided into multiple groups based on its structure but the function is always similar. Chicken-type or c-type lysozyme was first discovered in chicken albumen and has since become a very important medically and commercially protein. It works by splitting specific bonds in the peptidoglycan structure, which is the main component of the gram-positive bacterial cell wall. As a result, the cell wall breaks and the bacteria die. Other biological functions have been reported as well, but these are less well known. (Baker, 1974; B. Y. R. G. Board & Fuller, 1973; Wellman-Labadie et al., 2007)

Ovomucin is the protein responsible for the typical egg white gel-structure. Just like ovalbumin, it does not perform a direct antimicrobial function. Again, ovomucin-derived peptides have been found to perform antimicrobial activity (Wellman-Labadie et al., 2007).

Avidin is a trace component of the albumen but its function is well understood. This glycoprotein has a high affinity for biotin, a B-group vitamin necessary for survival. Each avidin molecule can tightly bind multiple biotin molecules, making them unavailable for bacterial use. (Abdou et al., 2013)

Table 1: Overview of the most abundant antimicrobial proteins identified in the chicken egg albumen. Based on information combined from Board & Fuller (1973) and Deeming & Ferguson (2003).

Protein	Contribution to albumen weight (%)	Molecular weight (kDa)
Avidin	0,1	68,3
C-type lysozyme	3,5	14,3
Ovalbumin	63,8	45
Ovomucin	1,4	230-3800
Ovomucoid	10,1	28
Ovotransferrin	13,7	77,7

An additional structure is often considered a part of the albumen. The chalaza encloses the yolk sac and connects it to both ends of the egg, keeping it in the middle when it egg is turned by the parent. This provides indirect protection by preventing the yolk to sink to the bottom where it would come in touch with the shell and have a much higher chance of infection. (Blackburn & Stewart, 2021; De Reu, 2006)

2.2.1.4 Yolk

The yolk is surrounded by the vitelline membrane which physically separates it from the albumen. It is important during the first four days of incubation, after which the embryonic membranes take over its function. It is of considerable strength because it forms a diffusion barrier between two liquids with a very different composition. Any leakage would cause the yolk and albumen to mix directly as a result of the difference in osmolarity, causing the failure of the egg. In addition, the vitelline membrane does not respire which indicates that there are little options for microbes to penetrate this structure without breaking it. (Bellairs et al., 1963)

Few antimicrobial proteins are detected in this structure. Lysozyme C proved to represent a considerable portion of the proteinaceous vitelline membrane. In addition, an ovomucin-like protein is also detected. Ovalbumin and ovotransferrin are expected, but not confirmed. (Mann, 2008)

The yolk itself is consists mainly of lipoproteins. Antimicrobial proteins found within the yolk are phosvitin, vitamin-binding proteins, and egg yolk immunoglobulin IgY (Abdou et al., 2013). These molecules increase the antimicrobial efficiency of the egg but are not as important as the rest of the chicken egg compartments.

2.2.2 Reptile egg structure

As far as reptiles are concerned, the structural diversity of eggs is much higher than found in birds. The vast majority of oviparous reptiles produce eggs with some sort of protective shell, although its structure is quite diverse and is traditionally classified as either being flexible and mostly proteinaceous as in squamates (Figure 5, C) to being rigid and highly calcified in crocodiles and turtles (Packard and DeMarco 1991). Although recent studies suggest that this classification needs to be reassessed given that much variation ranges along a continuum between these two groups (D'Alba et al., 2021).More importantly, non-avian reptile eggs are seldomly studied and their antimicrobial defence mechanisms remain largely unexplored. Most studies focus on the development of the embryo, contain no more than two or three species or focus only on one particular structural factor. To provide an overview of the current knowledge, the structural properties of the major clades are briefly discussed separately. Figure 4 provides the taxonomic relationship of the major reptilian clades and the type of egg they produce.

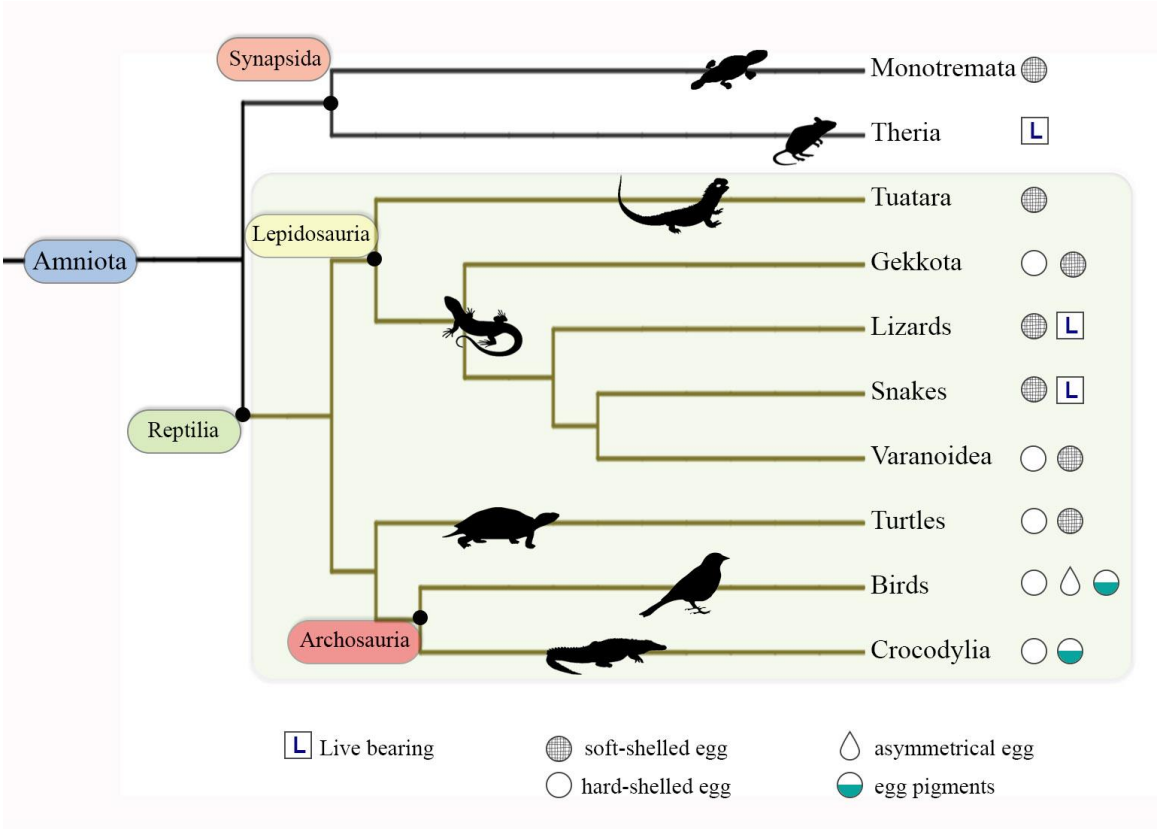

Figure 4: Simplified cladogram illustrating relationships between mayor clades of amniotes and the type of egg they produce.

2.2.2.1 Tuatara

The oldest clade is the Sphenodontida (Tuatara), endemic to New Zealand. The order arose during the Triassic period and includes only one living remnant. The reproduction rate is slower than that of any other reptile observed. It takes the female up to 3 years to produce the yolk and an additional 6 to 8 months to form the shell, no albumen is found within their eggs. In addition, the flexible-shelled eggs have an incubation period of 12 to 13 months. (Newman et al., 1994)

The Spenodontidan egg is unique when compared to other reptiles The eggshell consist of a multi-layered fibrous membrane and a thin, crystalline matrix in the form of calcite. The calcified layer is composed of separate columns that root deeply into the underlying membrane. (M. J. Packard et al., 1982)

2.2.2.2 Squamata

Squamata is by far the largest reptilian clade, representing 96,3% of the non-avian reptile diversity. The order includes more than 9100 species, making it one of the most successful vertebrate clades existing today (Pincheira-Donoso et al., 2013). Members are distributed, though highly unevenly, over the Serpentes (snakes), Lacertilia (lizards) and Amphisbaenia (worm lizards) (Pincheira-Donoso et al., 2013). Squamate eggs are very diverse, but most members lay parchment-shelled eggs (Figure 5). The shell consists of a fibrous membrane with a thin calcareous layer in the form of calcite. This layer often lies as a crust on the membrane or exists as isolated deposits (Deeming & Ferguson, 2003). Eggshell type is correlated with different life-history strategies and weakly influenced by phylogeny (Hallmann & Griebeler, 2015). All squamate eggs are common in that they entirely lack albumen (Girling, 2002).

2.2.2.3 Turtles

In the Testudines, flexibility in the eggshells is determined by the relative thickness of the calcareous layer. The shell units are made of aragonite and extend a short distance into the shell membrane, thereby providing attachment to the latter. In flexible-shelled eggs, the units are small and irregular whereas in rigid-shelled eggs, these units abut closely and form a highly organised, dense layer. Intermediate forms exist and have shell units ranging in between the characteristics described above (Figure 5). (Deeming & Ferguson, 2003). As far as albumen is concerned, the diversity is similar and various amounts have been detected inside turtle eggs (Girling, 2002).

2.2.2.4 Crocodylia

The crocodilian lineage is the most closely related to the avian clade. Their eggs have many similarities when compared to birds (Figure 5). The shell is highly calcified and contains clear pores that lead to the relatively thin shell membrane (Deeming & Ferguson, 2003). The yolk is surrounded by a thick layer of albumen that is thought to have the same functional characteristics as observed in birds (Girling, 2002).

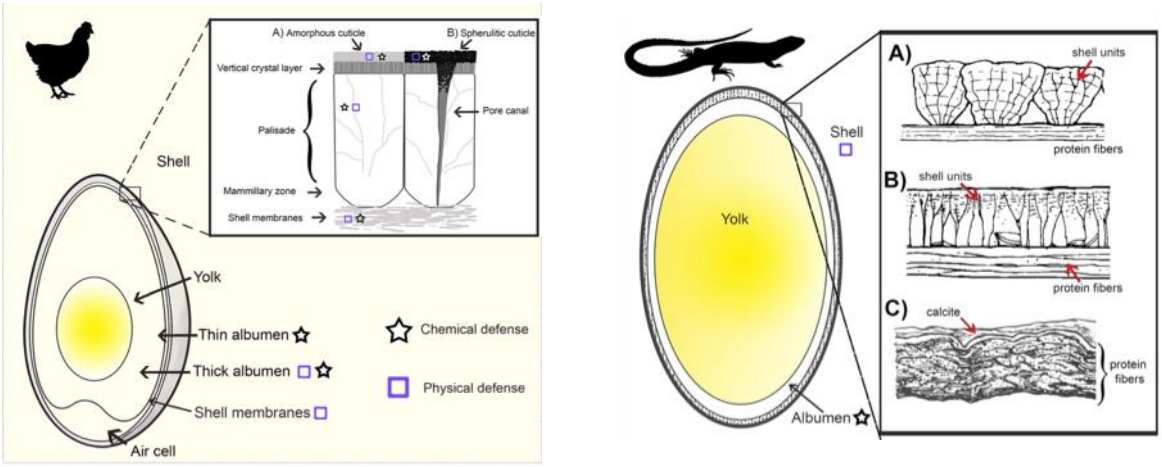

Figure 5 Comparison of antimicrobial defences in avian (left) and reptilian (right).

Shell structure (physical defence) is more complex and thicker and albumen (chemical defence) is more extensive in avian eggs. Left: A) Crocodylian shell, B) Gekkotan shell, C)

2.3 PROBLEM DEFINITION

From an evolutionary point of view, bird eggs are seen as more derived than their non-avian counterparts. They are described as cleidoic because they depend on their environment for respiration only. In general, the microenvironment is strictly regulated by their parents due to incubation. Parental incubation creates an optimal environment that has little temperature fluctuations and, as a direct consequence, a low humidity (D'Alba et al., 2010). Humid conditions increase the frequency of trans-shell infection and the high temperature stimulates the proper functioning of the antimicrobial proteins (Horrocks et al., 2014). As a result, unincubated eggs have a higher risk of microbial contamination (Cook et al., 2005).

The situation is entirely different in non-avian reptiles. As opposed to birds, reptiles seldomly incubate their eggs. Despite the potential importance of microbial infection for the survival and performance of individuals and populations, studies describing and identifying microflora affecting the eggs of reptiles are rare. Yet, both bacteria and fungi have been isolated from reptile nests and eggs (Wyneken et al., 1988; Phillot and Parmenter 2001) and are known to reduce the hatchling success of sea turtle eggs (Sarmiento-Ramírez et al., 2014) caimans (Nuñez Otaño et al., 2014) and lizards (Moreira & Barata, 2005). Also, many eggs rely on moisture for the successful development of the embryo and gain water from their environment throughout this period (M. Packard et al., 1982; Stahlschmidt et al., 2010). In addition, reptile eggs generally have a longer incubation period, extending the time frame in which microbes can enter the egg.

These factors indicate that the eggs of non-avian reptiles are under an increased selective pressure in terms of antimicrobial efficiency. However, when looked at their egg morphology, they seem to lack all the properties that, to our current understanding, promote protection of amniote eggs from microbial infection.

3 AIM

The aim of this research is to gain more insight into the mechanisms that protect the eggs of non-avian reptiles against microbial contamination. The goal is to get a better understanding of the overall efficiency as well as to find out what components of the egg are able to inhibit growth and/or kill microorganisms.

To reach this goal, reptile eggs will be analysed in structure, composition and performance. The results will be compared subsequently to the better-known avian eggs, in particular, the egg of the domestic chicken. This will be done descriptively over diverse phylogenetic groups to cover a substantial amount of variability between and within taxonomic groups.

4 MATERIALS AND METHODS

4.1 SAMPLE COLLECTION

Reptile eggs were donated by ZOO Antwerpen (Antwerp, Belgium) in Spring and Summer of the year 2020 and in April of the year 2021. Eggs from a ground nesting bird (crested partridge, *Rollulus* roulroul) were obtained from the Pakawi Park (Olmen, Belgium) in February 2021. In March 2021, free-range eggs were collected from non-commercial domestic chickens from a local private breeder in Belgium. To prevent microbial proliferation, all eggs were stored in the freezer at a temperature of -20°C until further use. In total, eggs were used from 21 different avian and non-avian reptile species (Appendix, Annex 1).

4.2 GENERAL INFORMATION

Information about nesting ecology of each species was obtained from the database included in D'Alba et al. (2021) and from species-specific articles. Nest type, temperature, average incubation time, breeding season, average clutch size and humidity were included in our dataset.

To estimate the volume of the eggs, a standardised picture was taken and analysed using the package 'Egg Tools' (Troscianko, 2014) within the ImageJ Software.

Frozen eggs were weighted to the nearest 0,1 g using a digital balance (KERN PLJ-C) and carefully cracked open. To weigh the different components of the eggs, the yolk, albumen and shell were separated while the egg remained frozen using a scalpel, then weighted and stored in sterile plastic tubes per egg component.

4.3 CHARACTERISATION OF EGGSHELL STRUCTURE AND EGG COMPOSITION

4.3.1 Shell

4.3.1.1 Histology

Microbes can easily digest proteins but not mineralized tissues. Therefore, I aimed to characterize the major components of the reptile shells. Individual fragments of shells were embedded, sectioned, stained and observed using light microscopy. Hard-shelled eggs were first treated with EDTA for 15 days to enable histological procedures.

A small piece of each shell was carefully cut out and embedded with epoxy Resin by gradually increasing the concentration. The embedded samples were trimmed and cut in sections of 3,5 µm thickness, using a Leica UC-6 ultra- microtome (Leica Microsystems, Germany).

After drying, the sections were stained using three different procedures separately. Alizarin red S was applied for 2 minutes to reveal the amount of mineral deposition of the shell. It selectively stains calcium, visualising aragonite and calcite in red (Dickson, 1966). Alcian blue was used to locate glycoproteins, which are known to be a major component the extracellular matrix of the eggshell membrane in chicken eggs (Kaweewong et al., 2013). The dye specifically targets acidic mucopolysaccharides and was applied for 15 minutes and washed away with deionised water. The last staining procedure is the Masson's Trichome staining which visualises keratin in pink and collagen and mucin in light green.

The sections were observed and digitally recorded using light microscopy (Leica DM1000 optical microscope plus attached digital camera Leica DMC 4500). The resulting pictures were analysed with the ImageJ software to measure and calculate the relative composition of the shells (i.e. proportion mineral, protein and polysaccharides).

4.3.1.2 Scanning electron microscopy

Scanning electron microscopy was used to analyse the gross topography, structure, thickness and inorganic-organic distribution of materials present in the shells.

From each species, three small pieces were carefully cut from the eggs and mounted on an aluminium stub to visualise the outer surface, inner surface and cross section respectively. The samples were sputter-coated them with gold/palladium for 2 minutes and imaged on a SEM (FlexSEM 1000; Hitachi) at an accelerating voltage of 10 kV and 6 mm working distance.

The micrographs were analysed with the ImageJ software to quantify the parameters of interest.

4.3.2 Albumen and yolk

I encountered certain limitations of frequent access to some lab facilities and/or training personnel and therefore the performance of specific tests, namely the quantification and identification of proteins in egg components. Therefore, I selected a subsample of taxonomically distinct species to perform these tests. I hypothesized that species that do not deposit albumen in their eggs, produce yolks with as strong a antimicrobial capacity as eggs which contain albumen. therefore, in selecting the samples for the following tests, I ensured than an equal representation of eggs with and without albumen were considered in the set.

The same yolk-extracts and albumen samples of the previously described tests were used to increase accuracy and enable comparison between performance and proteins present.

4.3.2.1 Protein concentration

The protein concentrations were measured using the Bradford protein assay (Bradford, 1976). This procedure makes use of a brown to blue colour shift that occurs when Bradford reagent binds to proteins. By measuring the shift in absorbance, the protein concentration can by calculated by including a standard curve of known concentration.

The standard curve was produced by making a dilution series of Bovine Serum Albumin (BSA) with increasing concentration from 0 to 2 mg/ml. Glycine buffer was used as diluent to be consistent with the yolk-extracts. 5 µl of the protein samples and BSA dilutions were added, in triplicate, to a 96-wells plate. In each well, 250 µl Bradford reagent was added and spectrophotometrically analysed at 595 nm (Bio-Rad 680XR) for 5 minutes.

4.3.2.2 Lysozyme activity quantification

Lysozyme is an enzyme found in avian egg white as well as many mammalian secretions (milk, tears, saliva) (Baker, 1974) and therefore expected to be universal among tetrapod species. It works by lysing the cell wall of a wide range of gram-positive bacteria, making it a non-species specific antibacterial enzyme. As a result, the activity of lysozyme, or any enzyme with a similar function, can be measured by monitoring the decrease of turbidity of a bacterial suspension, over time. This technique is based on the work of Shugar D. (1952).

The analysis of potential lysozyme activity in the content of the eggs, is based on the modified turbidimetric assay of Liao et al. (2001). This procedure makes use of a solution of *Micrococcus lysodeikticus* dry cell walls. When lysozyme is added to the suspension, the cell walls break,

causing a decrease in turbidity over time. This decrease can be quantified using a spectrophotometer.

The suspension was made by dissolving 25 mg of dry cell walls of *Micrococcus lysodeikticus* ATCC 4698 (Sigma-Aldrich) in 50 mL of sodium phosphate buffer (0,1 mM, pH 6,2). In addition, a standard lysozyme solution was made by adding 10 µg of chicken egg white lysozyme (Sigma-Aldrich) to 1 mL of phosphate buffer (0,1 M, pH 7,0). Just before the experiment, a twofold serial dilution was made, starting from the standard lysozyme solution.

To analyse the performance of the eggs, 10 µl of each egg sample (yolk-extract or albumen) in triplicate, were added to a 96-wells plate, 10 µl of each of the tubes containing the lysozyme serial dilution (in duplicate) were added to the first and second rows of the plate. Then, 150 µl of *Micrococcus* solution were added to each well and immediately put into the spectrophotometer for analysis of absorbance. The absorbance was recorded at 560 nm using a plate reader (Wallac 1420 VICTOR 2 Perkin Elmer, MA) at 1-minute intervals for 20 minutes. The concentration of lysozyme in each sample was calculated by comparison to the reduction in absorbance (OD) between the initial and final timer, obtained by the lysozyme standard curve.

4.3.2.3 Protein identification

4.3.2.3.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe the weight of the proteins present and gain a first insight into the (dis)similarities between different samples.

After the concentration was determined, a certain volume of each protein was put into separate tubes so that it contained 50 µg proteins. Laemmli buffer was added and the mixture was loaded into the gel (12,5% Tris-HCl) together with a molecular weight marker (Bio-Rad, precision Plus Protein™ Unstained Standard). One lane included the Laemmli buffer only to serve as a blank. The gel ran vertically at 140V until the front line almost reached the end of the gel. Thereafter, the gel was removed and stained over night with Coomassie Blue G while being shaken. The gel was rinsed with 30% methanol to remove the remaining dye.

4.3.2.3.2 MALDI-TOF MS

To identify the proteins, MALDI-TOF mass spectrometry was used. The stained protein bands were cut out of the previously made gel, with a clean scalpel, and put into sterile 1,5 ml Eppendorf tubes.

To remove the Coomassie Blue G stain, 150 µl of 200 mM NH_4HCO_3 in 50% Acetonitrile was added to the tubes and incubated at 30°C for 20 minutes. The liquid was removed and the procedure was repeated until all the samples were completely decoloured. To remove the remaining liquid, the gels slices were dried using a speedvac concentrator (model SPD121P, Savant SpeedVac™) and cooled on ice.

To cut the proteins, 10 µl of digest buffer was added to ice-cooled slices and further incubated on ice for 45 minutes. The buffer consists of 50 mM NH_4HCO_3, pH 8,5, containing 0,002 µg modified trypsin (50x enzyme stock of 0,1 µg trypsin/µl, Promega Trypsin kit, Madison, WI; USA, cat. N° V5111). Thereafter, the gel slices were dilutes with 40 µl of digest buffer and incubated overnight at 37°C.

The supernatant, containing the alkaline peptides, was collected in a new, clean 0,5 ml Eppendorf tube and 0,1% formic acid was added. The acidic peptides were obtained from the

gels slices by a two step extraction. This was done by adding 40 µl of 60% acetonitrile in 0,1% formic acid to the slices and incubated for 20 minutes at 30°C. After vortexing for three minutes, the supernatant was added to the alkaline peptides and the last step was repeated. The mixture was speedvac dried and the peptides were redissolved in 12 µl of 0,1% TFA – 50% acetonitrile.

The MALDI matrix α-cyano-4hydroxy cinnamic acid was prepared, using the ABSCIEX matrix from the MASS standards kit for calibration (AB Sciex TOF/TOF instruments, 4333604), according to the description included in the kit. The mixture was vortexed and centrifuged, both one minute. The resulting supernatant was used as matrix.

The peptide extracts were mixed with the matrix in a 1/1 ratio and 1 ml was spotted onto an Opti-TOF 384 Well MALDI Plate Insert (ABSCIEX, Framingham, MA 01701, USA, PN 1016629). The plate was put into the MALDI-TOF mass spectrometer (4800 Plus MALDI TOF/TOF™ Analyzer, SCIEX) to perform mass spectrometry followed by tandem mass spectrometry on the 5 highest peaks.

The resulting spectra were analysed using the ProteinPilot™ software together with MASCOT search engine (SCIEX, USA). To obtain results as correct as possible, different databases were obtained from Swiss-Prot (6-05-2021) for each taxonomic unit as narrow as possible (i.e. Crocodylia, Testudines, Serpentes, Iguania, *Gallus gallus*).

4.4 ANTIMICROBIAL PERFORMANCE

4.4.1 Chemical defence

To analyse the egg's ability to inhibit growth and/or kill microorganisms, tests were performed on the different components of the eggs. Chemical defence is rendered by proteins in the egg that interfere with the metabolism of microbes. Many of such proteins that are known to interact with a multitude of organisms and hence are referred to as non-species specific.

The albumen in birds is composed mainly of proteins and water. Assuming this is also true in those reptile eggs that contain albumen, no extraction of proteins is needed as such a mixture is relatively easy to use in the inhibition tests. The yolk, however, is highly variable between species in terms of viscosity. In addition, the large amount of lipids present, renders the yolk mostly solid with a butter-like texture. To increase accuracy of the amount of yolk used in the tests, the proteins of the yolk were extracted before testing. The protocol was followed described in Hernández De Morales et al., 1987. Briefly, yolk was added to 0.02 molar Glycine buffer in a sterile plastic tube. One litre of the buffer was prepared by adding 1.5 gram of Glycine and 0.006 gram of Sodium Hydroxide to 800 millilitre deionised water. After adding a few drops of HCL to achieve a pH of 8, deionised water was added until the buffer had a volume of exactly 1 litre and the solution was sterilised for 30 minutes by means of an autoclave. To extract the proteins, a volume of 3 millilitre of yolk was added to an equal volume of Glycine buffer. After thoroughly vortexing the mixtures, the tubes were centrifuged for 12 minutes at a speed of 11000 rounds per minute at room temperature. The supernatant contained the protein solution and was pipetted into a new, sterile plastic tube. Extracts were stored in the fridge at 6°C until use.

4.4.1.1 Disc diffusion-test

4.4.1.1.1 Yolk and Albumen

To analyse the antimicrobial efficiency of the yolk and albumen, standard disc-diffusion tests were performed. We selected bacterial strains as to include potential egg pathogens as defined in Grizard et al. (2014) and Shawkey et al. (2008). A selection was made to include species from a variety of taxonomic groups, assuming that closely related species could be inhibited by similar non-species specific antimicrobial mechanisms. In total, cultures of 12 bacteria and 2 fungi were used in this test. After growing a monoculture of the microbes, they were each put in solution until an optical density of 0.1 was reached. This was done using a sterile cotton swab, a glass tube with physiological water and an analog OD-reader to the nearest 0,01 ABU (BIOLOG) under sterile conditions. The solution was further diluted with a factor 10^{-2}. Thereafter, the microbial solution was swabbed onto a clean agar plate (see Appendix, Annex 2 for details) and a sterile disc was placed in the middle. The disc was made out of absorbent filter paper (Whatman, England) and had a diameter of 3 millimetre. Finally, a volume of 10 microlitre of yolk extract or albumen was pipetted on top of the disc and the plates were put into the incubator at 28 °C. One plate, per microbial species, contained a disc with physiological water to serve as a blanc. Microbial growth around the disc was observed and a picture was taken. This was repeated after one and 7 days of incubation. The plates were analysed using the ImageJ software.

4.4.1.1.2 Eggshell

A similar approach was used to test the chemical performance of the eggshell. However, extracting proteins from this structure has proven to be very difficult and could not be performed. Instead of using a disc, small pieces of shell of each species were put onto the plates. They were cut under sterile conditions and carefully rinsed clean with deionized water to minimize contamination. After an incubation period of respectively 1 and 8 days, microbial growth around the shell was assessed.

4.5 PATHOGEN IDENTIFICATION

4.5.1 Collection

One of the bird eggs was obtained from a destroyed and abandoned nest of a geese population living in an urban park (Citadelpark, Ghent, Belgium) and stored in the freezer. Upon taking an eggshell sample, the frozen, seemingly intact, egg exploded as the result of microbial infection. The full content was blackish-green and neither the albumen, neither the yolk were recognisable. A sample from the inside was put into a 1,5 ml Eppendorf tube and stored in the freezer at -20°C.

4.5.2 Growing

To identify a few microbes in the identified egg, 1 ml physiological water was added to the samples and a dilution series was made from 10^{-1} to 10^{-10}. Each dilution was swapped onto three sterile growth plates and incubated under the conditions presented in Table 2.

Table 2:Overview of the different growth conditions of samples of a contaminated goose egg.

Environment	Growth medium	Temperature (°C)	Oxygen condition
1	Columbia Blood Agar - 5% Sheep blood	20	Aerobe (air)
2	Columbia Blood Agar - 5% Sheep blood	37	Aerobe (air)
3	Reinforced Clostridia l Medium	37	Anaerobe

Growth was observed after 4 days and for each environment, the plate with the best separate colonies was chosen and colony forming-units were counted. From these three plates, 10 colonies were picked and streaked onto a new plate each, to obtain isolated cultures. This was done with a sterile inoculation loop of 10 µl.

After 24h of growth, the young subcultures were stored for identification through MALDI-TOF MS. From each subculture, 2 colonies were picked with a sterile inoculation loop of 1 µl, and diluted in 300 µl sterile milli-Q water. After this; 900 µl was ethanol was added to each tube for protein precipitation. The mixture was vortexed thoroughly, put in the centrifuge for 5 minutes and stored in the freezer at -80°C until further use.

4.5.3 MALDI-TOF MS

Prior to analysis, the samples were brought to room temperature. They were centrifuged for three minutes after which the supernatant was discarded. The tubes were centrifuged again and the remaining liquid was entirely removed with a pipet. After the pellets were airdried, formic acid and acetonitrile were added to resolubilize the proteins. The samples were spotted onto the target plate (Msp 96 Polished Steel Maldi Target Plate, Bruker) and covered with 0,5 µl α-cyano-4hydroxy cinnamic acid matrix.

After drying, the plate was put into the MALDI-TOF mass spectrometer (MALDI Biotyper, MBT smart, Bruker) for analysis. The spectra were automatically compared to the Bruker database and the LM-UGENT database by the MBT compass-software.

4.6 RESEARCH GROUPS

Experiments regarding the antimicrobial performance, protein concentration, protein identification and pathogen identification were performed in the Laboratory of Microbiology at Ghent University.

5 RESULTS

5.1 EGGSHELL STRUCTURE

5.1.1 Degree of mineralisation

In addition to defining the hardness of the shell, the percentage of calcium present seems to be relatively stable within the larger phylogenetic groups included in this study. It is important to note that these results should be interpreted carefully as the amount of samples per group is limited. Figure 6 shows an overview of the degree of mineralisation across the taxonomic groups included in this in research. In hard shelled eggs, the calcium content is clearly visible as a dense layer in the form of tightly packed crystals. In soft-shelled eggs, the presence of crystals was mostly observed at the outer surface of the shell or as stained dots, distributed across the protein matrix.

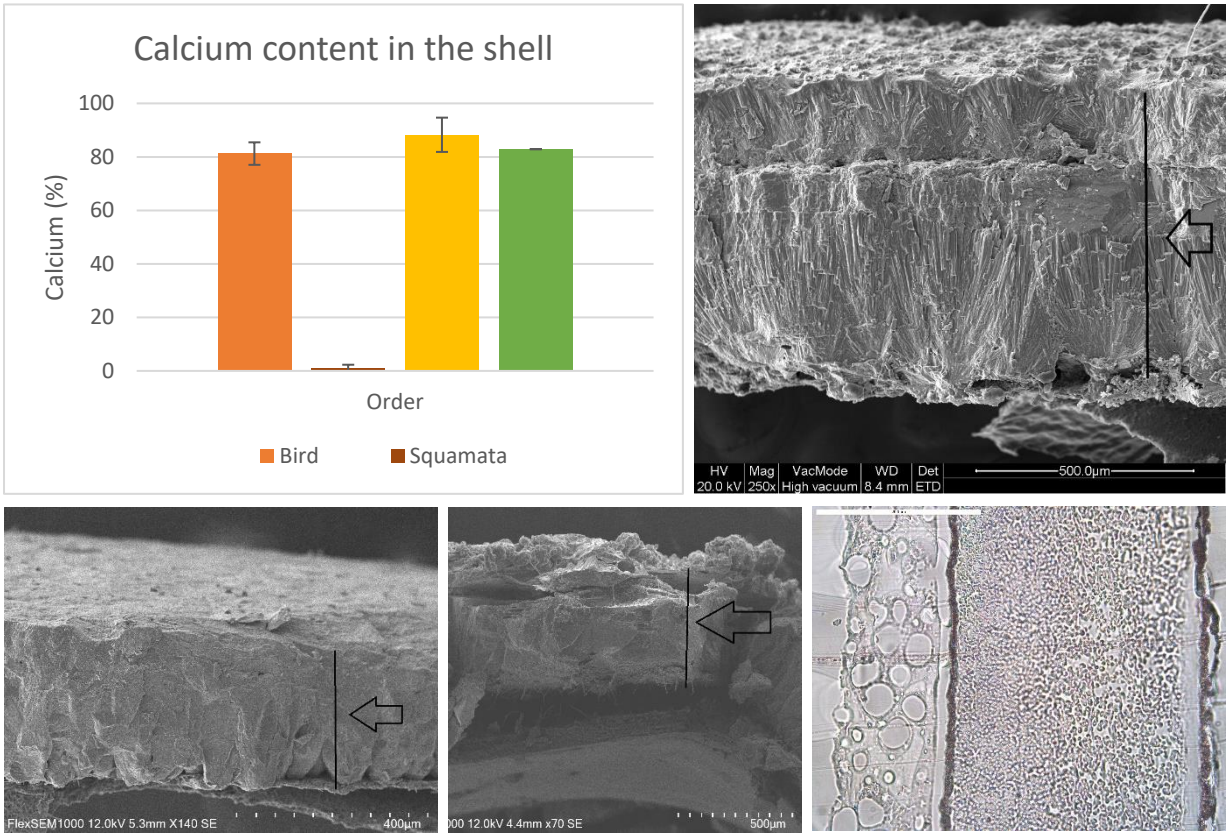

Figure 6: Calcium content in the shell in different taxonomic groups.
Top: Graph representing the comparison between groups (left) and SEM picture of a turtle shell (right).
Bottom: SEM pictures of the shells of bird (left) and crocodile (middle) and a microscopic picture of a squamate shell stained with Alizarin red.

Interestingly, micrographs of *Cuora amboinensis* revealed strong microbial proliferation on its eggshell. The microbial growth on the eggshell was evident as long “twisted’ fibers and ovoid bodies identified as fungi hyphae and spores respectively (Figure 7). The fungal structures were observed on the outer and inner surfaces of the shell.

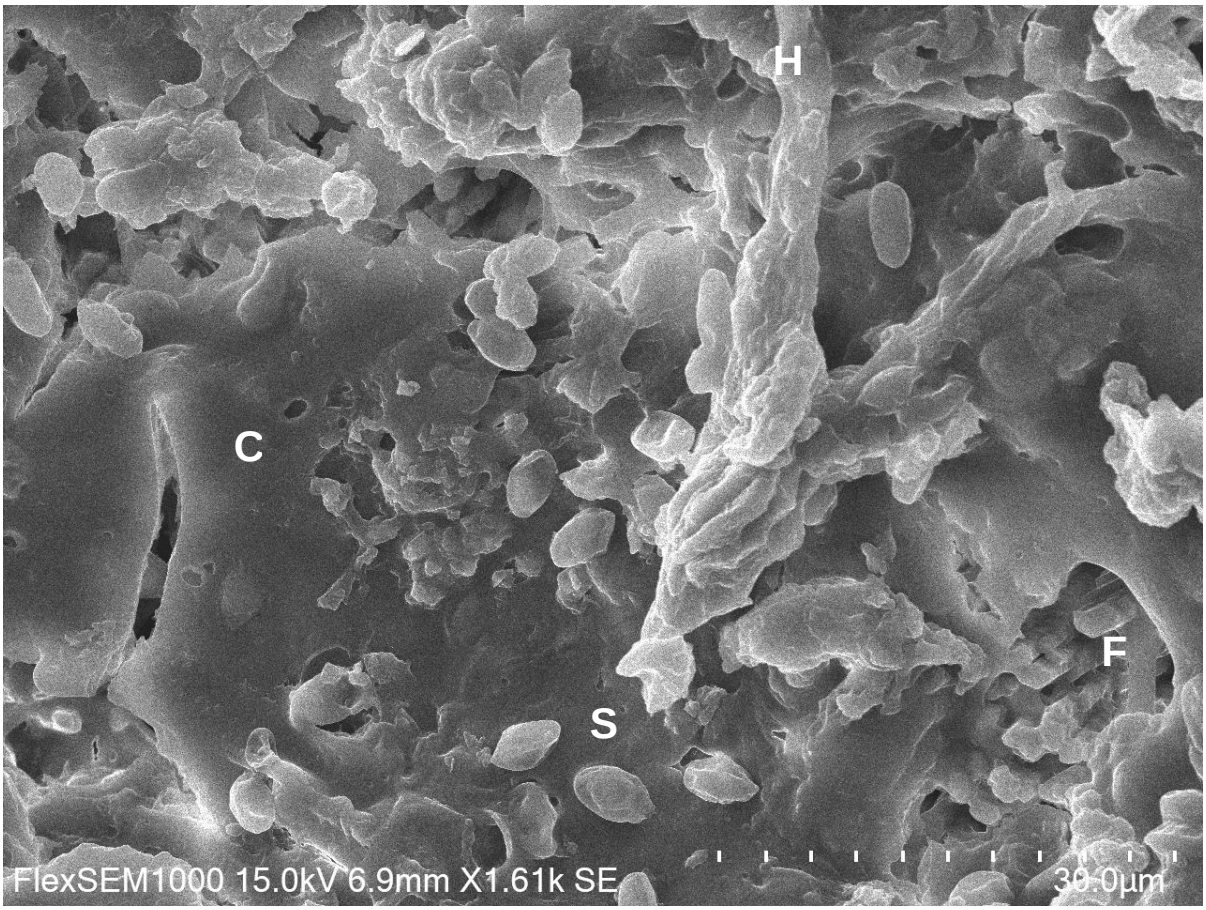

Figure 7: Micrograph of the outer surface of a turtle (Cuora amboinensis) eggshell showing pervasive fungal growth. The fungal hyphae (H) and ovoid spores (S) can be seen interspersed the shell fibres (F) and shell cover (C).

5.1.2 Proteins

The amount of organic proteins present in the shell varies substantially between species. Nevertheless, two major patterns can be observed. First, in almost all “soft” shelled samples stained, the proteinaceous elastic tissue is most abundant, followed by glycoproteins and collagen and mucus respectively (for exact numbers, see Appendix, Annex 3).

Second, soft shells contain much more proteins when compared to the shell membrane of hard shells. All protein groups tested showed a difference between these two morphologically distinct egg types (Figure 8), with collagen and mucus almost entirely absent in hard-shelled eggs. In some turtle species, these protein group is located within the mineralised crystal as a matrix (Figure 8, b).

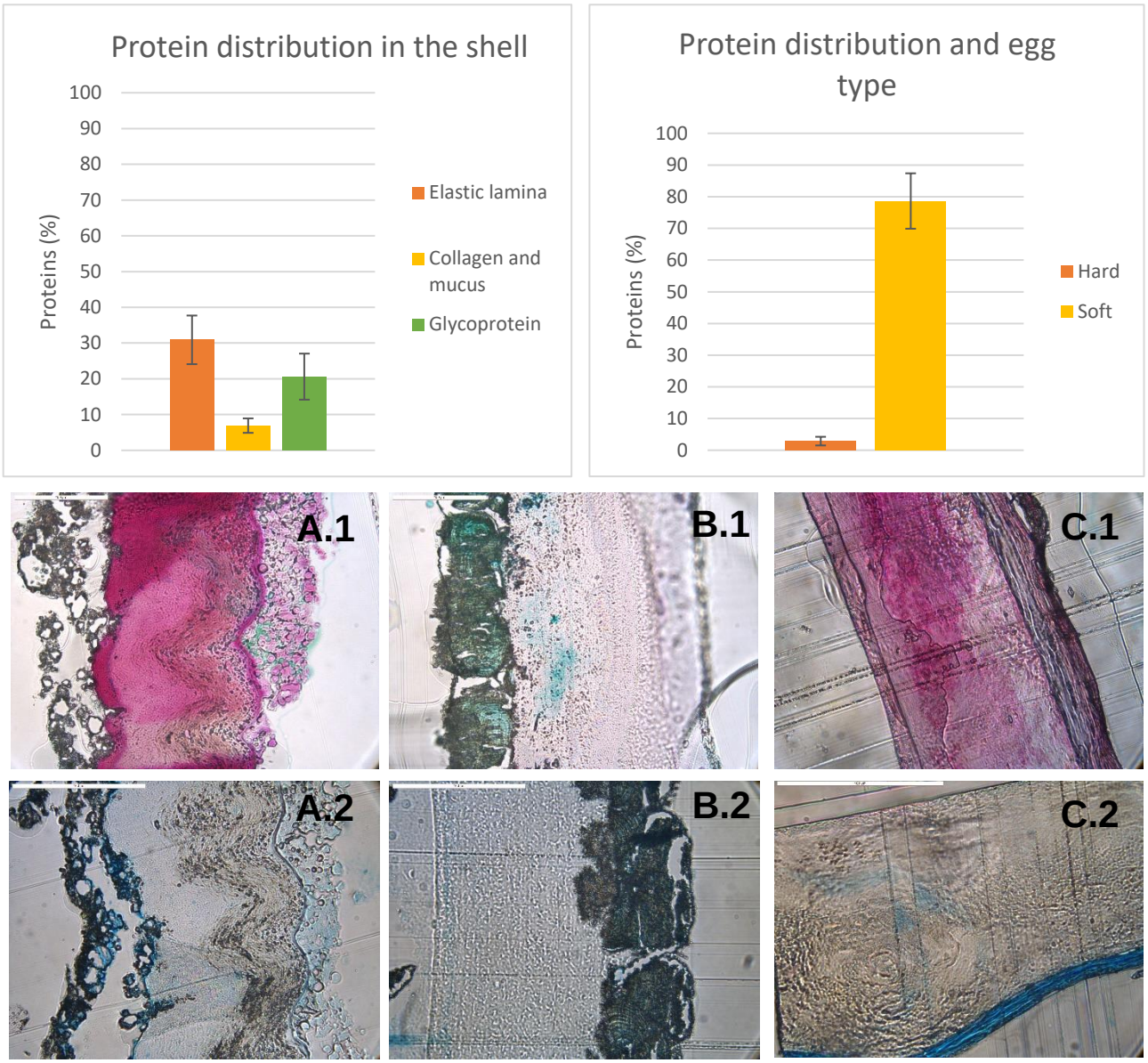

Figure 8: Relative abundance of the different proteins present in in the shell.
Top: The average amount over all the shells analysed (left) and the overall abundance based on shell type (right).
Bottom: Microscopic pictures of shell slices of Sauromalus obesus (A), Pelusios species (B) and Caimain crocodillus (C). after Masson's Trichome staining (1) and Alcian blue (2)

5.2 EGG COMPOSITION

5.2.1 Protein content

I found that in eggs that contain both, yolk and albumen, the protein concentration is much higher in the yolk. On the other hand, in soft-shelled eggs, the protein concentration in the yolk exceeds that of is hard-shelled counterparts (Figure 9). See Appendix, Annex 4 for exact values.

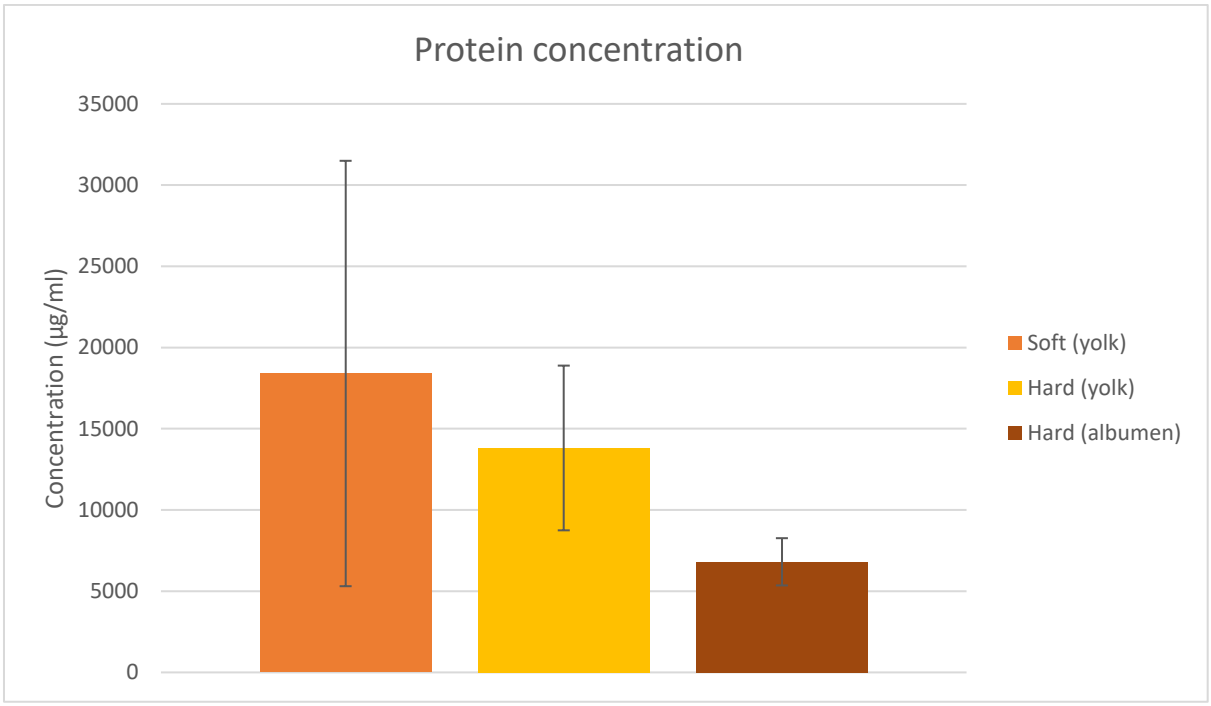

Figure 9: Total protein concentration of the different egg components measured using the Bradford reagent.

5.2.2 Lysozyme concentration

The lysozyme concentration, determined by the reduction in optical density, is shown in Figure 10. A lot of variation was observed between the samples with some OD-reductions below the detection limit (i.e. *Hydrosaurus weberi, Pelusios species* and *chelonoidis carbonaria*), while others exceeded the calibration curve (i.e.*Gallus gallus, Elseya branderhorsti, Morelia spilota* and *Sauromalus ater*) (see Appendix, Annex 5 for the results).

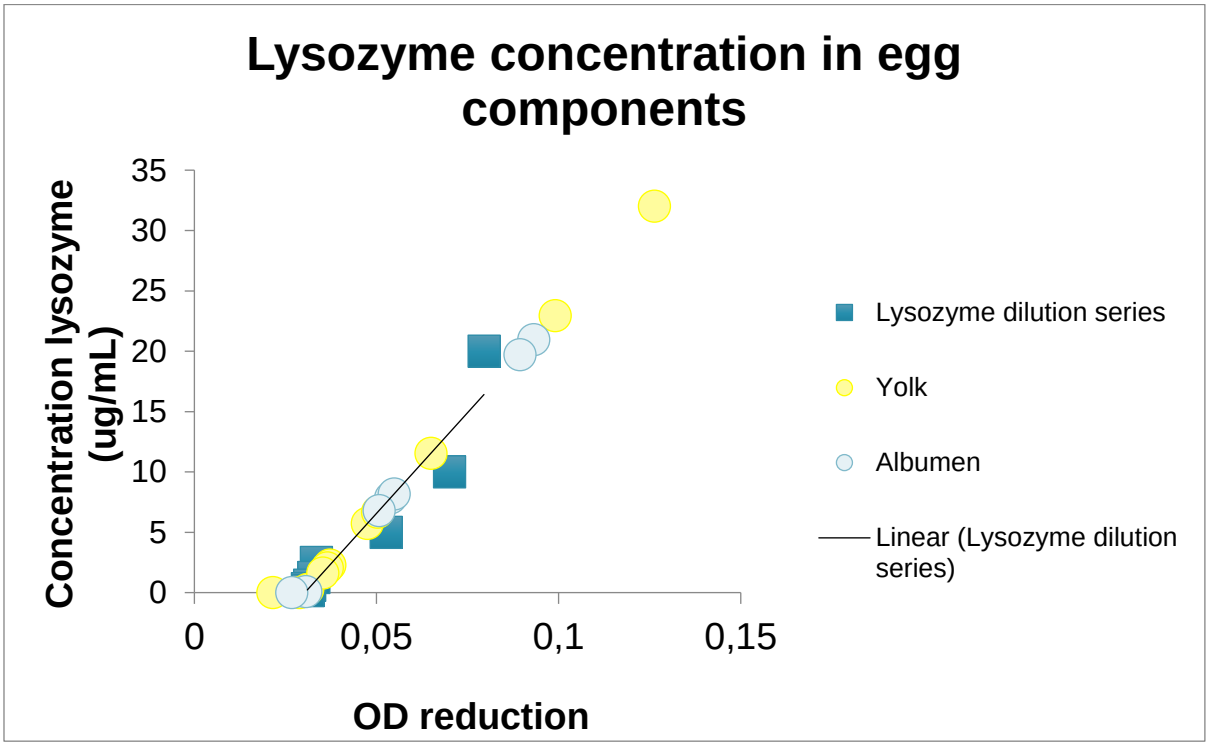

Figure 10: Lysozyme concentration of the different egg compartments determined by the reduction in optical density.

In general, the lysozyme concentration found in the soft-shelled eggs was higher than lysozyme in the yolk and albumen in hard-shelled eggs although extreme values were found in both compartments (Figure 11).

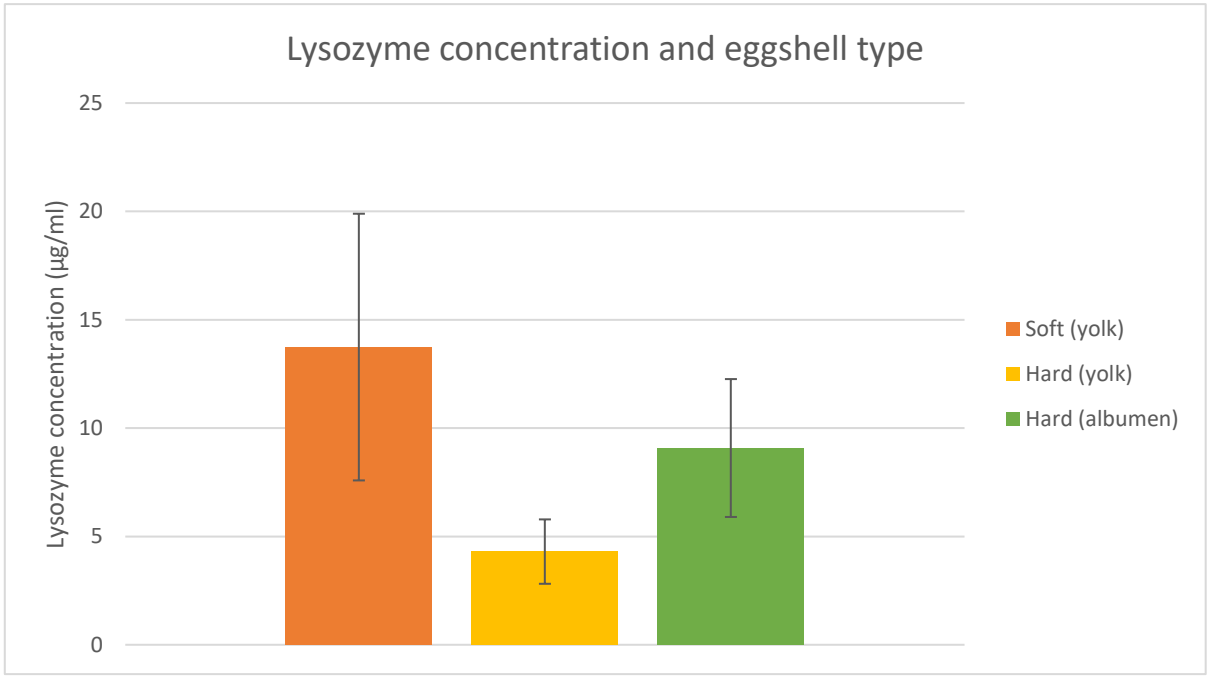

Figure 11: Lysozyme concentration observed in the egg components divided by egg type.

5.3 ANTIMICROBIAL DEFENCE

5.3.1 Disc-diffusion

In general, the inhibitory activity of egg components was very low or null for most species (see Appendix, Annex 6 for numeric values). The results of the inhibition activity of the egg components against 12 microbial strains are shown in Figure 13.

The strength of inhibition is presented by green and orange, indicating a strong and weak reaction respectively. The inhibition was strong when the direct area around the disc was clear of bacterial colonies. Weak inhibition was considered when the clear area was very small or when microbes showed considerably smaller colony-forming units close to the disc, relative to those observed in the rest of the plate. No inhibition was evident when the bacterial growth completely surrounds the disc (Figure 12).

Nevertheless, a few patterns can be observed. The results are relatively similar between samples across the different taxonomic groups. No inhibition ever occurred against fungi (i.e. *Candida* and *Aspergillus*) and some bacterial strains (i.e. *Micrococcus, Phenylobacterium, Sphingomonas, Escherichia,* and *Vibrio*) included in the experiment. In general, the antimicrobial performance of the yolk was larger when compared to the albumen, both numerical and in frequency. Aside from the two bird species and one turtle (*Elseya branderhorsti*), reptilian albumen showed no inhibition at all. Finally, the eggs of turtles and birds seem to be more resistant over a broader range of bacterial genera when compared to the eggs of crocodiles and squamates.

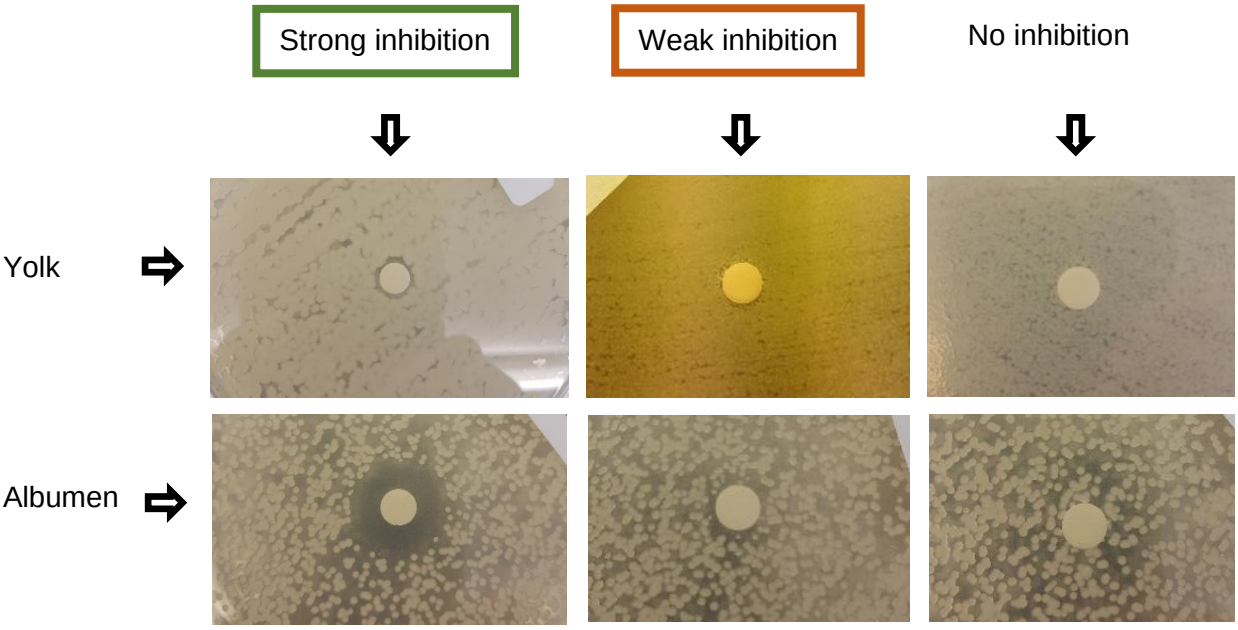

Figure 12: Overview of the interpretation of the strength of inhibition.
Top: Inhibition observed by the yolk, the results show Bacillus (left) and Citrobacter (middle and right).
Bottom: Inhibition observed by the combination of Bacillus and albumen.

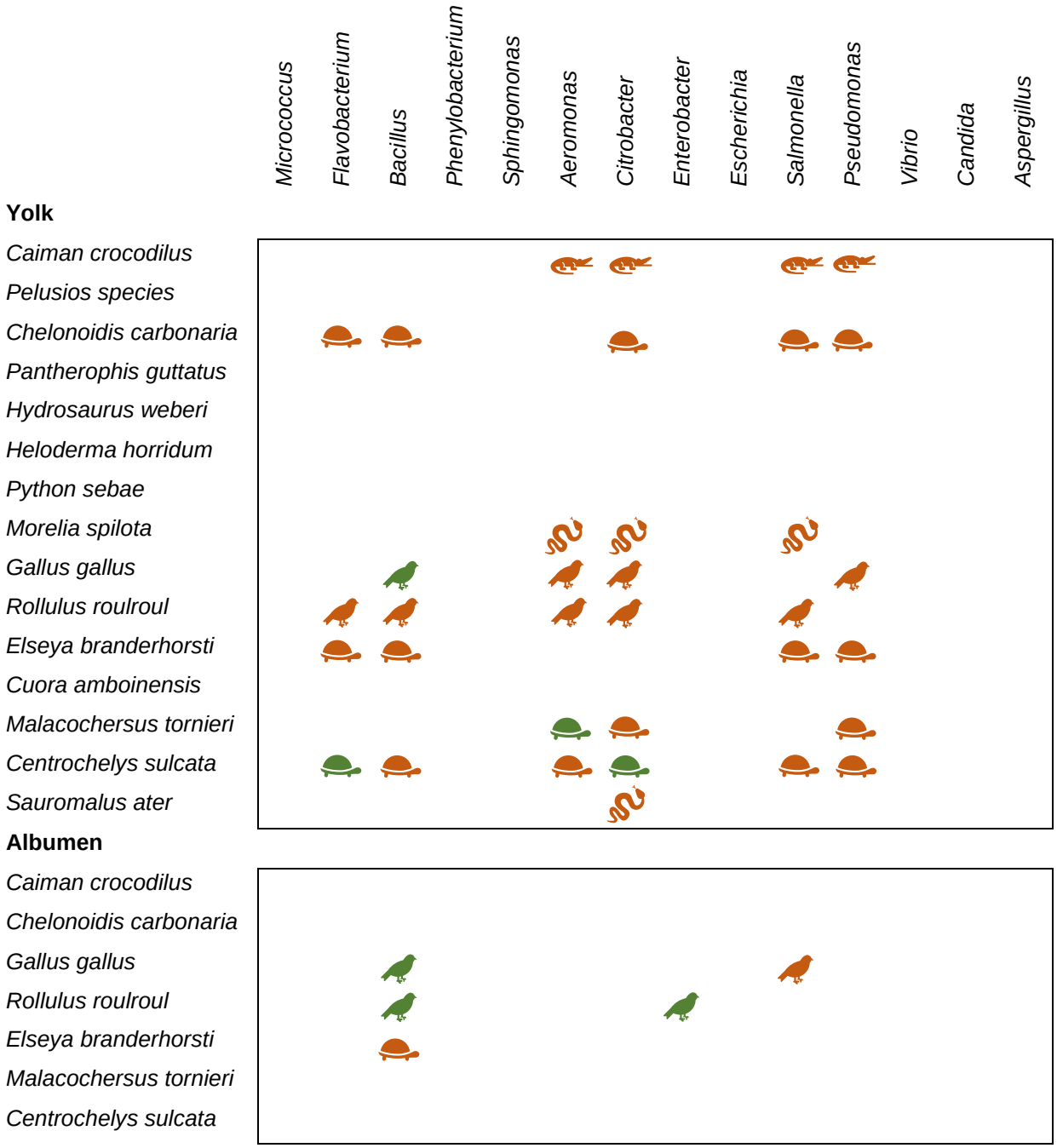

Figure 13: Schematic representation of the results of the disc-diffusion test. The pictograms of the crocodile, turtle, snake and bird represent members of the Crocodylia, Testudinata, Squamata and Birds respectively. Strong and weak inhibition is shown in green respectively.

5.3.2 Shell

No analyses of the shells could be made because all samples were highly contaminated, after incubation with the growth medium, resulting in microbial proliferation on the incubated plates.

5.4 PROTEIN IDENTIFICATION

5.4.1 SDS-PAGE

5.4.1.1 Yolk

At first inspection, many the protein patterns in the yolk look quite similar within taxonomic groups (Figure 14). The bands of the two bird species are nearly identical, based on molecular weight. The crocodilian egg yolk resembles the avian pattern with clear bands present at around 150, 100, 60, 37 30 and between 10 and 15 kDa. Turtle egg components also show similar bands to avian and crocodile eggs (150, 100, 60, and 37 kDa) and show strong within-clade similarity. The squamate eggs break this pattern, although they do show some similarities with the other clades (100 and 60 kDa).

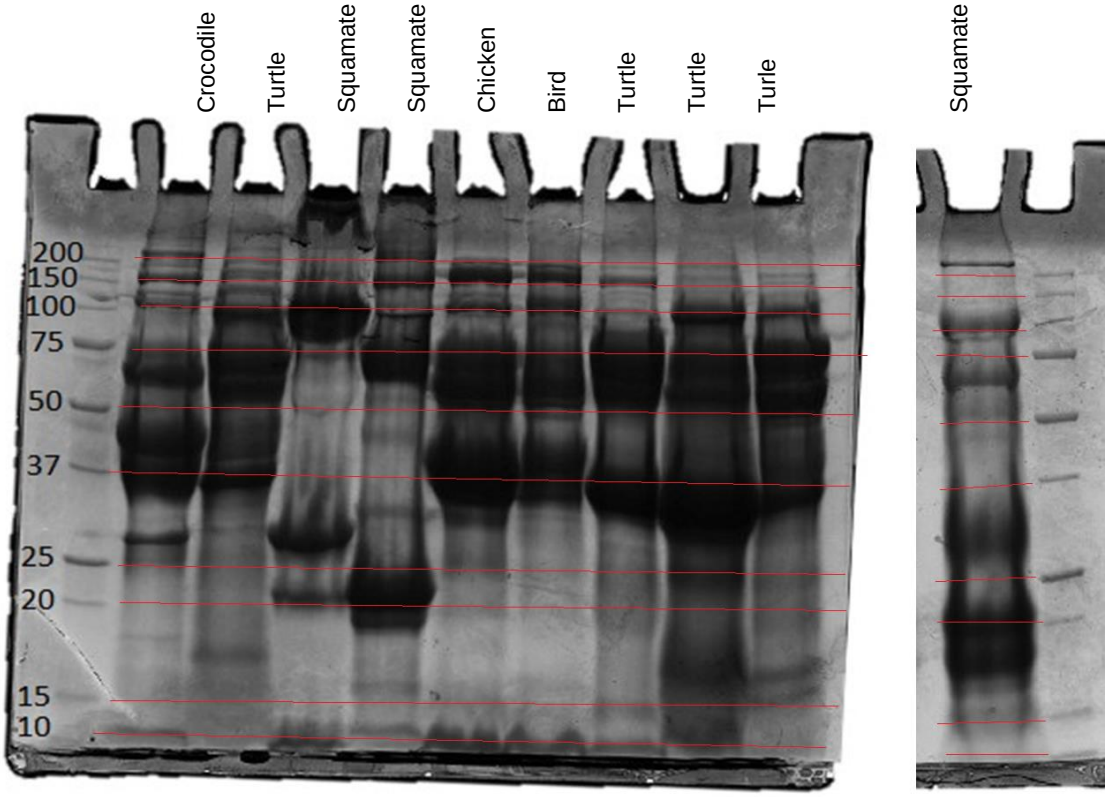

Figure 14: Scanned graph of the SDS-PAGE gel loaded with yolk-extracts. The proteins were stained with Coomassie Blue and are visible as dark bands according to their molecular weight in kDa.

5.4.1.2 Albumen

When looking at the chicken albumen, the well known proteins are clearly visible (Figure 15). Ovalbumen is by far the most abundant one and can be seen by the immense amount present around 45 kDa. Avidin is visible at around 68 kDa. In addition, c-type lysozyme (14,4 kDa) can be seen as a clear, separate lane at the bottom of the gel. These molecular weights are also visible in the other avian sample, although less clear.

The turtle samples look very similar to one another with clear bands visible at 150, 100, 75, 37, 30, ~17 and ~12 kDa. The crocodile shows a similar pattern aside from the absence of proteins at around 17 kDa and the clear presence at around 50 kDa.

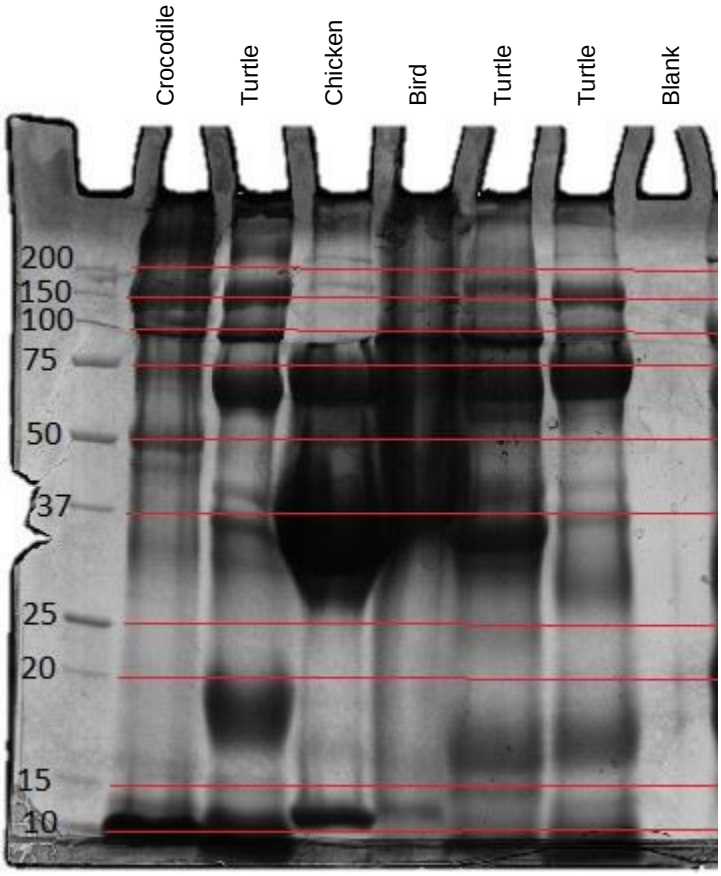

Figure 15: Scanned graph of the SDS-PAGE gel loaded with Albumen. The proteins were stained with Coomassie Blue and are visible as dark bands according to their molecular weight in kDa.

5.4.2 MALDI-TOF MS

Due to the similarities observed in the SDS-PAGE analysis, a selection was made of protein bands with at different molecular weights.

Unfortunately, the MALDI-TOF characterisation did not lead to any significant match. One problem proved to be the scarcity of reptile egg proteins in the data base. Nevertheless, a few proteins matched with antimicrobial proteins found in frogs (Table 3)

Table 3: Overview of the protein matches observed by MALDI-TOF MS in a subsample of protein bands, cut out of the gels.

Sample	Component	Weight (kDa)	Match
Crocodile	Albumen	~75	Plasticin-B1
Turtle	Yolk	25-30	Dybowskin 2-CDYa
Turtle	Yolk	~37	Xenoposin-precursor fragment B1
Squamate	Yolk	~100	Riparin-1.1
Squamate	Yolk	~12	Distinctin-like peptide
Turtle	Albumen	~37	Palustrin-3a

5.5 PATHOGEN IDENTIFICATION

The results of the analysis are shown in Table 4. Interestingly, multiple genera were isolated from the seemingly monocultural, second generations of plated bacteria (Appendix, Annex 7).

Enterococcus and *Bacillus* grew in all and two out of three conditions respectively. Aerobe conditions at 37°C contained the highest microbial diversity.

Table 4: Overview of the bacterial species present in a contaminated goose egg, identified with MALDI-TOF MS.

Environment	Identified species
Aerobe 20°C	*Bacillus pumilus* *Enterococcus casseliflavus*
Aerobe 37°C	*Bacillus pumilus* *Enterococcus casseliflavus* *Enterococcus mundtii* *Lysinibacillus xylanilyticus*
Anaerobe 37°C	*Enterococcus casseliflavus* *Enterococcus mundtii*

6 DISCUSSION

In this study, the main aim was to get an overview of the antimicrobial efficiency of non-avian reptile eggs and draw comparisons with the better known avian eggs. I predicted that soft-shelled eggs would show a stronger antimicrobial efficiency relative to hard-shelled eggs. The results of this study were consistent with this prediction, although many questions remain.

I observed a large interspecific variation in the shells analysed so far. Soft shells in particular, showed a wide diversity in eggshell thickness and structural composition. Together with the variety in protein distribution, these results suggest a much larger structural diversity, both between and within groups, than previously expected from the general physical characteristics. This large variation could be an indication that shell characteristics are closely related to the habitat in which they are laid. Reptile eggs rely much more on their direct environment when compared to birds, particularly in terms of hydric conditions. Thus, this hypothesis should be considered in future studies. Unfortunately, I did not find sufficient variation in the bactericidal capacity of egg contents to test the association between specific egg components and antimicrobial efficiency. For example, it could be expected that eggs with higher protein content and less mineralized, would show stronger antimicrobial efficiency.

Nevertheless, interesting general patterns in the antimicrobial performance and eggshell type were found. For example, I found that the yolk of soft-shelled eggs outperformed the components of hard-shelled eggs. This is consistent with the idea that the first are more prone to contamination, given that the eggshell, as physical barrier, is in general more permeable. Thus, soft-shelled eggs would have to rely on stronger chemical defence mechanisms. Interestingly, reptile eggs that contain both yolk and albumen all showed a larger inhibition performed by the yolk. This could indicate that the albumen does not necessarily serve as protection against microorganisms in non avian reptiles as it does in avian eggs (Board and Tranter, 1995).

The overall defence efficacy of all egg components was unexpectedly low, except for the activity of the chicken albumen against the *Bacillus* strain. However, as described by Prajanban et. al (2012), ovotransferrin seems to be the main antimicrobial protein present in reptilian eggs. It works by causing an iron depletion in the albumen, preventing microbes from taking it up. The organisms were grown on a specifically chosen growth medium, to cause them to flourish without much inhibition. Many bacteria were able to form colonies closely to the injected discs. Small colonies were often found a short distance from the disc, increasing in size with increasing distance. This pattern could indicate that the observed inhibition is the result of iron depletion but is not effective when combined with an optimal growth medium. However, these patterns were sample specific and repeated experiments should be performed to validate this theory. Moreover, many strains seems to be fully unaffected by the presence of the egg compartments. The most unexpected outcome was the unaffected *Micrococcus* strain, which is known to be highly effected by the presence of lysozyme. However, the same strain was used in all experiments and lysozyme resistance is known to occur frequently (Ragland & Criss, 2017).

Moreover, lysozyme concentration differed widely between the samples. Although the results were consistent with the rest of the findings, additional experiments should be performed to support this idea. For example, the conditions in which different lysozymes are active can differ widely between organisms with optimal temperatures ranging from 20°C up to 80°C (Lee et al., 2007; Miyazaki, 1998; Kim et al., 2012).

The highly similar protein distributions in the yolk and albumen was also expected. Aside from antimicrobial proteins, amniote eggs are full of highly conserved proteins, regulating and facilitating embryonic development. Nevertheless, the most abundant protein in reptile albumen would correspond with ovotransferrin (Prajanban et al., 2012), at a molecular weight of 75kDa. A band corresponding with that molecular weight occurred in the turtle eggs observed in this study. In addition, lysozyme exists in multiple isoforms and the c-type is known to be present in the chicken egg and has previously been identified in turtle and crocodile species (Prajanban et al., 2012). This molecule has a mass of 14,4 kDa and seems to be present in these species as well as with ovalbumin, which weighs around ~45 kDa in the albumen of non-avian reptiles (Prajanban *et. al,* 2012).

Unfortunately, precise identification of such proteins was unsuccessful. In general, very few proteins have been fully identified in amniote eggs, other than birds and such information is almost entirely absent for the eggs of non-avian reptiles. Unfortunately, as the result of the COVID-19 virus, I encountered technical difficulties associated with maintenance of the MALDI-TOF mass spectrometer. This caused the device to decrease in accuracy, leading to very few peaks to be analysed by MS-MS. One chicken albumen sample was included, specially cut out of the gel were lysozyme c-type was expected to be, according to the molecular marker. Although this sample was in fact matched with chicken lysozyme, the results were not significant. Additional matches were observed, although not significant. The matches with antimicrobial proteins found in frogs could indicate that there are similarities with the active domain of these proteins. Although this is merely a speculation, this observation forms an interesting path for further investigation.

Lastly, the pathogens identified in the infected eggs were more or less as expected. *Enterococcus* is known to be pathogenic in birds and is ubiquitous in the nesting environment, especially in the avian gut. By contrast, the presence of gram-positive bacteria (i.e. *Bacillus* and *Lysinibacillus*) regularly found in forest soil and humus (Lee et al. 2010) and with pathogenic activity was interesting because it gives insight into the source of contamination in eggs. Their presence in the egg contents also suggest that, given the level of contamination I observed, albumen was no longer recognisable and might have been entirely depleted of lysozyme and strongly degraded.

7 CONCLUSION

This is the first study to the best of our knowledge to analyse the antimicrobial efficiency over a wide range of non-avian reptile species. Exploring the different egg components and comparing them to those of birds has rendered interesting results.

The overall findings seem to indicate that the eggs of non-avian reptiles contain multiple factors to protect the embryo from damage due to microbial infection. However, additional research is necessary to reveal the identity and functioning of these mechanisms present. A tremendous diversity exists within the eggs of the reptilian clade and learning more about their form-function relationship would reveal crucial insights that are relevant for multiple disciplines, such as ecology, microbiology, biomimicry and evolution.

8 ABSTRACT

8.1 ENGLISH

Reptiles form a very successful clade with over 20 thousand currently living species. One of the most important key factors causing this great diversity is the amniote egg. The amniote egg allowed early reptiles to develop a fully terrestrial life cycle, making them able to colonize land and adapt to new environments.

Amniote eggs are characterised by a large yolk that is a very nutritious food source, not only for the growing embryo, but for almost all life forms on Earth. Predation has been recognized as the major cause of egg mortality. The relatively thick eggshell forms the first layer of physical protection and effectively prevents many macropredators from reaching the coveted content of the egg. Strikingly, it is much less clear how reptiles are able to deal with predation by microorganisms. Developing embryos do not have an immune system yet and unlike mammals they cannot rely on antibodies rendered by the mother. In addition, microbes are known to be very abundant in nesting environments and infection can cause the egg to fail.

As opposed to birds, many non-avian reptile eggs rely on moisture for the successful development of the embryo and gain water from their environment. These factors indicate that the eggs of non-avian reptiles are under an increased selective pressure in terms of antimicrobial efficiency. However, when looked at their egg morphology, they seem to lack all the properties that, to our current understanding, promote protection of amniote eggs from microbial infection. The aim of this research is to gain more insight into the mechanisms that protect the eggs of non-avian reptiles against microbial contamination.

Eggshell structure is observed using histological staining combined with optical microscopy. The composition of the egg is studied by testing its antimicrobial performance and analysing its protein content and identity. In addition, MALDI-TOF mass spectrometry is used to identify a subset of pathogenic bacteria present in a highly contaminated egg with a seemingly intact shell.

The results of this research indicate that the eggs of non-avian reptiles contain multiple factors to protect the embryo from damage due to microbial infection. A high variety exists in the different components but the present findings seem to highlight the great need for a deeper investigation of the optimal conditions conductive to full functionality of antimicrobial egg defences.

8.2 NEDERLANDS

De Reptilia vormt een zeer succesvolle clade met meer dan 20 duizend bestaande soorten. Een van de belangrijkste elementen van deze grote diversiteit is het amniote ei. Dankzij het amniote ei konden vroege reptielen een volledig terrestrische levenscyclus ontwikkelen, waardoor zij het land konden koloniseren en zich aan nieuwe omgevingen konden aanpassen.

Kenmerkend voor deze eieren is de grote dooier, die een zeer voedzame voedingsbron vormt, niet alleen voor het groeiende embryo, maar voor bijna alle levensvormen aanwezig op aarde. Predatie is erkend als de belangrijkste oorzaak van eiersterfte. De relatief dikke eierschaal vormt de eerste laag van fysieke bescherming en voorkomt dat veel macropredatoren de begeerde inhoud van het ei kunnen bereiken. Opvallend is heersende onduidelijkheid over hoe reptielen in staat zijn om te gaan met predatie door micro-organismen. Ontwikkelende embryo's hebben nog geen immuunsysteem en in tegenstelling tot zoogdieren kunnen zij niet terugvallen op antilichamen die door de moeder worden aangemaakt. Bovendien is bekend dat microben zeer talrijk zijn in nestomgevingen en dat infectie het ei kan doen falen.

In tegenstelling tot vogels zijn veel eieren van strikte reptielen afhankelijk van vocht voor een succesvolle ontwikkeling van het embryo en halen zij water uit hun omgeving. Deze factoren wijzen erop dat de eieren van reptielen onder een verhoogde selectieve druk staan wat betreft de antimicrobiële efficiëntie. Wanneer we echter kijken naar hun ei-morfologie, lijken ze alle eigenschappen te missen die, volgens onze huidige inzichten, de bescherming van het amniote ei tegen microbiële infectie bevorderen. Het doel van dit onderzoek is meer inzicht te krijgen in de mechanismen die de eieren van strikte reptielen beschermen tegen microbiële besmetting.

De structuur van de eierschaal wordt geobserveerd met behulp van histologische kleuring gecombineerd met optische microscopie. De samenstelling van het ei wordt bestudeerd door de antimicrobiële werking ervan te testen en het eiwitgehalte en de identiteit ervan te analyseren. Bovendien wordt MALDI-TOF massaspectrometrie gebruikt om een subset van pathogene bacteriën te identificeren die aanwezig zijn in een sterk besmet ei met een schijnbaar intacte schaal.

De resultaten van dit onderzoek wijzen erop dat de eieren van reptielen meerdere factoren bevatten om het embryo te beschermen tegen schade door microbiële infectie. Er bestaat een grote variëteit in de verschillende componenten, maar de huidige bevindingen lijken te wijzen op de grote behoefte aan een dieper onderzoek naar de optimale omstandigheden die leiden tot een volledige functionaliteit van antimicrobiële afweermechanismen.

9 SUMMARY IN LAYMEN TERMS

Reptile eggs are often referred to as nature's most perfect design. The ability to breath air while protecting the developing embryo is a very intriguing combination for multiple reasons.

Aside from the domestic chicken, little is known about the mechanisms that render protection of the egg against the ubiquitous microorganisms that could cause the egg to fail. The eggs of non-avian reptiles in particular rely much more on a proper balance between egg characteristics and environmental conditions. However, when looked at their morphology, they seem to lack all the necessary tools, present in birds that are known to perform this task.

The aim of this research is to gain a better understanding of the ongoing processes that result in antimicrobial protection. We found that the eggs of reptiles are highly variable in all levels of organisation. They seem to be better equipped in terms of antimicrobial efficacy when compared to birds. Increasing our knowledge about the mechanisms that enable reptiles to develop in microbe-rich environments could reveal interesting insights in a broad range of biological fields and could potentially lead to technological innovations, designed through biomimicry.

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12 APPENDIX

Annex 1: General overview of the species included in this research.

Species name	Common name	Egg-type	State	Obtained
Caiman crocodilus	Spectacled caiman	Hard	Complete	AntwerpZoo
Pelusios sp.	African mud turtles	Soft	Complete	AntwerpZoo
Sauromalus obesus	Common chuckwalla	Soft	Complete	AntwerpZoo
Chelonoidis carbonaria	Red-footed tortoise	Hard	Complete	AntwerpZoo
Anser anser domesticus	Domestic goose	Hard	Complete	Wild
Pantherophis guttatus	Corn snake	Soft	Complete	AntwerpZoo
Hydrosaurus weberi	Weber's sailfin lizard	Soft	Complete	AntwerpZoo
Eublepharis macularius	Common leopard gecko	Soft	Eggshell	AntwerpZoo
Naja annulifera	Snouted cobra	Soft	Complete	AntwerpZoo
Pogona sp.	Bearded dragon	Soft	Eggshell	AntwerpZoo
Python regius	Ball python	Soft	Eggshell	AntwerpZoo
Heloderma horridum exasperatum	Mexican beaded lizard	Soft	Complete	AntwerpZoo
Python sebae	African rock python	Soft	Complete	AntwerpZoo
Morelia spilota	Diamond python	Soft	Complete	AntwerpZoo
Gallus gallus domesticus	Domestic chicken	Hard	Complete	Garden
Rollulus rouloul	Crested partridge	Hard	Complete	AntwerpZoo
Elseya branderhorsti	Branderhorst's snapping turtle	Hard	Complete	AntwerpZoo
Cuora amboinensis	Amboina box turtle	Hard	Complete	AntwerpZoo
Malacochersus tornieri	Pancake tortoise	Hard	Complete	AntwerpZoo
Centrochelys sulcata	African spurred tortoise	Hard	Complete	AntwerpZoo
Sauromalus ater	Chuckwalla	Soft	Complete	AntwerpZoo

Annex 2: Overview of the growth media used to cultivate the microbial strains used in the disc-diffusion test.

Genera	Growth medium
Micrococcus	Nutrient agar
Flavobacterium	Nutrient agar
Bacillus	Nutrient agar
Phenylobacterium	Nutrient agar
Sphingomonas	Reasoner's 2A agar
Aeromonas	Nutrient agar
Citrobacter	Nutrient agar
Enterobacter	Nutrient agar
Escherichia	Nutrient agar
Salmonella	Nutrient agar
Pseudomonas	Nutrient agar
Vibrio	Iron Sulphate agar
Candida	Nutrient agar
Aspergillus	Nutrient agar

Annex 3: Percentage of the different proteins present in the eggshell. Results obtained by histological staining

Species	Elastic lamina (%)	Collagen and mucus (%)	Glycoproteins (%)
Caiman crocodilus	0,00	0,00	0,73
Pelusios species	37,31	21,80	26,63
Sauromalus obesus	61,57	14,88	35,19
Chelonoidis carbonaria	1,55	0,00	9,25
Anser anser	0,00	0,00	17,69
Pantherophis guttatus	43,58	3,48	0,00
Hydrosaurus weberi	12,42	12,42	58,30
Eublepharis macularius	39,10	9,35	64,70
Naja annulifera	81,80	11,85	2,38
Pogona species	41,10	1,55	45,93
Python regius	41,73	21,45	57,55
Heloderma horridum	64,75	0,00	3,81
Python sebae	9,13	3,24	4,79
Morelia spilota	29,52	3,79	0,00
Gallus gallus	0,00	0,00	0,00
Rollulus rouloul	0,00	0,00	0,00

Annex 4: Protein concentration present in the yolk and albumen.

Species	Yolk (µg/ml)	Albumen (µg/ml)
Caiman crocodilus	15030,790	5754,825
Chelonoidis carbonaria	4549,450	4920,259
Pantherophis guttatus	44586,670	No albumen
Morelia spilota	5642,932	No albumen
Gallus gallus	16320,220	14578,150
Rollulus roulroul	41999,400	4924,480
Elseya branderhosrsti	9878,738	/
Malacochersus tornieri	3518,492	6086,112
Centrolyches sulcata	5414,448	4585,764
Sauromalus ater	4973,998	No albumen

Annex 5: Summary of the lysozyme concentration of the yolk-extracts and the albumen, determined by the reduction in optical density.

Species	OD-reduction	Lysozyme (mµ/ml)
Yolk extract		
Caiman crocodilus	0,037	2,28141981
Chelonoidis carbonaria	0,036	2,0360272
Hydrosaurus weberi	0,022	under limit
Morelia spilota	0,099	22,9321645
Gallus gallus	0,065	11,5435582
Rollulus rouloul	0,047	5,72161796
Elseya branderhorsti	0,050	6,65177053
Malacochersus tornieri	0,031	0,25718444
Sauromalus ater	0,126	32,0068925
Centrochelys sulcata	0,035	1,61744109
Pelusios species	0,029	under limit
Albumen		
Caiman crocodilus	0,054	7,84884752
Gallus gallus	0,093	20,9638045
Rollulus rouloul	0,055	8,16397073
Elseya branderhorsti	0,089	19,7118854
Centrochelys sulcata	0,051	6,79468389
Chelonoidis carbonaria	0,031	0,07489664
Malacochersus tornieri	0,027	under limit

Annex 6: Area in mm that should inhibition around the disc of the disc-diffusion experiment.

	Micrococcus	*Flavobacterium*	*Bacillus*	*Phenylobacterium*	*Sphingomonas*	*Aeromonas*	*Citrobacter*	*Enterobacter*	*Escherichia*	*Salmonella*	*Pseudomonas*	*Vibrio*	*Candida*	*Aspergillus*
Yolk														
Caiman crocodilus	0	0	0	0	0	0,8	1,3	0	0	1,5	1,3	0	0	0
Pelusios species	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chelonoidis carbonaria	0	7,0	1,0	0	0	0	4,9	0	0	0,8	5,9	0	0	0
Pantherophis guttatus	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
Hydrosaurus weberi	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heloderma horridum	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
Python sebae	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
Morelia spilota	0	0	0	0	0	4,5	0,6	0	0	2,5	0	0	0	0
Gallus gallus	0	0	56,7	0	0	1,5	1,3	0	0	0	4,9	0	0	0
Rollulus rouloul	0	2,9	3,7	0	0	3,9	2,7	0	0	2,5	0	0	0	0
Elseya branderhorsti	0	4,7	2,9	0	0	0	0	0	0	1,1	2,7	0	0	0
Cuora amboinensis	0	0	0	0	0	0	0	0	0	0	0	0	0	NA
Malacochersus tornieri	0	0	0	0	0	10,9	0,6	0	0	0	4,7	0	0	0
Centrochelys sulcata	0	14,3	2,1	0	0	3,7	9,8	0	0	2,1	1,9	0	0	0
Sauromalus ater	0	0	0	0	0	0	3,9	0	0	0	0	0	0	0
Albumen														
Caiman crocodilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chelonoidis carbonaria	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gallus gallus	0	0	120	0	0	0	0	0	0	4,3	0	0	0	0
Rollulus rouloul	0	0	63,0	0	0	0	0	9,8	0	0	0	0	0	0
Elseya branderhorsti	0	0	1,0	0	0	0	0	0	0	0	0	0	0	0
Malacochersus tornieri	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Centrochelys sulcata	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Annex 7: Results of the bacterial identification by mass spectrometry. A Bruker score larger than 2 means that the match is significant and the specimen can be identified with certainty to the genus level.

Environmental conditions	Identified species	Bruker score
Aerobe - 20°C	Enterococcus casseliflavus	2.13
	Enterococcus casseliflavus	2.29
	Enterococcus casseliflavus	2.1
	Enterococcus casseliflavus	2.16
	Bacillus pumilus	2.21
	Bacillus pumilus	2.17
	Enterococcus casseliflavus	2.14
	Enterococcus casseliflavus	2.09
	Enterococcus casseliflavus	2.13
	Enterococcus casseliflavus	2.14
	Enterococcus casseliflavus	2.14
	Enterococcus casseliflavus	2.11
	Enterococcus casseliflavus	2.13
	Enterococcus casseliflavus	2.13
	Bacillus pumilus	2.23
	Bacillus pumilus	2.18
	Bacillus pumilus	2.04
	Bacillus pumilus	2.14
	Enterococcus casseliflavus	2.11
	Enterococcus casseliflavus	2.14
Aerobe – 37°C	Enterococcus casseliflavus	2.1
	Enterococcus casseliflavus	2.14
	Enterococcus mundtii	2.55
	Enterococcus mundtii	2.52
	No Organism IdentificationPossible	1.28
	No peaks found	0.0
	Enterococcus casseliflavus	2.01
	Enterococcus casseliflavus	2.12
	Lysinibacillus xylanilyticus	2.23
	Lysinibacillus xylanilyticus	2.28
	Enterococcus casseliflavus	2.14
	Enterococcus casseliflavus	1.88
	Enterococcus casseliflavus	2.13
	Enterococcus casseliflavus	2.03
	Bacillus pumilus	1.93
	No Organism IdentificationPossible	1.56
	Enterococcus casseliflavus	1.96
	Enterococcus casseliflavus	2.12
	Enterococcus casseliflavus	2.21
	Enterococcus casseliflavus	2.19
	Enterococcus casseliflavus	2.1
	Enterococcus casseliflavus	2.06

Anaerobe – 37°C	Enterococcus casseliflavus	2.17
	Enterococcus casseliflavus	2.18
	Enterococcus mundtii	2.44
	Enterococcus mundtii	2.48
	Enterococcus mundtii	2.49
	Enterococcus mundtii	2.5
	Enterococcus casseliflavus	2.2
	Enterococcus casseliflavus	2.14
	Enterococcus mundtii	2.49
	Enterococcus mundtii	2.43
	Enterococcus mundtii	2.44
	Enterococcus mundtii	2.41
	Enterococcus mundtii	2.47
	Enterococcus mundtii	2.4
	Enterococcus casseliflavus	2.13
	Enterococcus casseliflavus	2.18
	Enterococcus casseliflavus	2.2
	Enterococcus casseliflavus	2.11