Water loss under controlled humidity and temperature regimes in relation to bird eggshell thickness

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Dissertation accepted in fulfilment of the requirements for the degree *Master of Science in Environmental Sciences* at the North-West University

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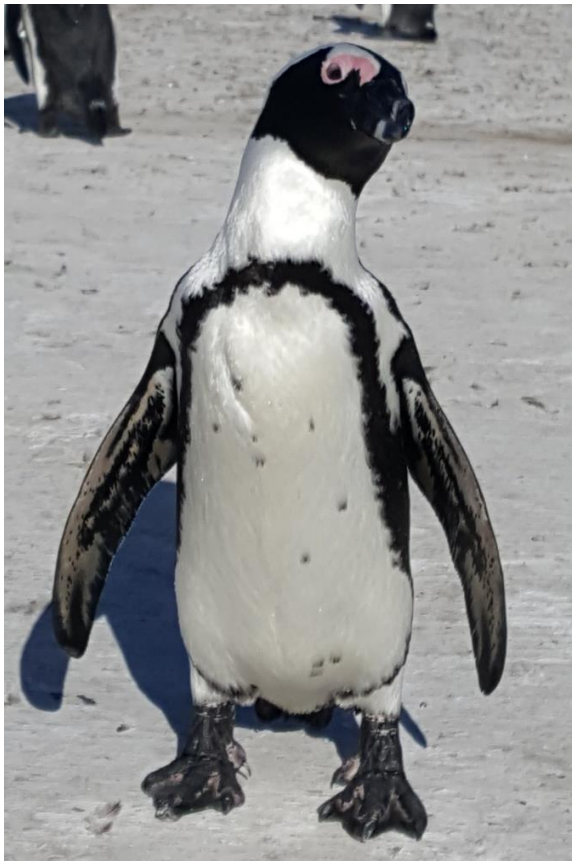
Graduation July 2020

24930024

Acknowledgements

- First, I am very grateful to Prof. Hindrik Bouwman for his support, assistance, and patience throughout this study. Thank you for making this possible.
- I also want to thank Petrus Bronkhorst for his help in making sure all the equipment was in working condition.
- Thank you, because this study was supported by the North-West University and CHIETA through their generous grants that helped me to make my dream into a reality.
- A big thanks to Willie Landman who helped me with the SEM, and Lourens Prinsloo who helped me with the Raspberry pi.
- Everyone else who endured me during this time with love and support especially Ricardo Botha.
- Lastly, I want to thank my examiners for their time and effort, especially for having to read the term “relative humidity” 96 times.



“Saving one animal won’t change the world, but it will change the world for that one animal.”

-Unknown author

ABSTRACT

The African Penguin (*Spheniscus demersus*) is an Endangered species occurring along the Indian and South Atlantic coasts of South Africa. There are many suspected causes for the precipitous decline in their numbers. Previously, eggshell thinning associated with increased concentrations of persistent organic pollutants has been found for the African Penguin. The combination of thinner shells and higher temperatures due to global climate change may cause accelerated water loss through the eggshells, thereby possibly harming the embryo.

A method had to be developed to measure water loss across eggshells under controlled temperature and humidity treatments. This I did in a previous study where I converted a chicken egg incubator, and successfully tested the effectiveness using chicken eggshells. Thinner eggs lost water faster than thicker shells at higher temperatures, moderated by humidity.

I used the African Penguin eggshells from a previous POPs study to measure the effects of temperature and humidity on water loss. I chose temperature and humidity treatment based on current climate data, and selected two higher temperatures (32°C and 37°C) and commensurate humidity settings to simulate future climate change scenarios.

The eggshells lost water faster at higher temperatures, and less water was lost at higher relative humidities irrespective of temperature, as was the case for chicken eggshells. However, eggshell thickness did not play a role. Thinner and thicker eggs lost water at the same rate at all temperature and humidity treatments. Water loss across the eggshells was therefore regulated by the shells, irrespective of their thickness.

I noticed from the data that the standard deviations increased at higher temperatures. Plotting the percentage coefficient of variation (%CVs) of the water loss at each relative humidity against their respective temperatures showed that the water loss regulation (low %CVs) was lost at around 32°C, and increased towards 37°C. An optimal 15°C temperature window for effective water loss regulation was indicated by the model.

Since previous studies have not investigated the combination of temperature, humidity, and eggshell thickness in relation to water loss, this study suggests an optimal temperature/humidity window related to water loss. Not all shells responded in this way, suggesting a variability upon which natural selection can act.

One additional factor was tested and that was the effect of the oily eggshell extraneous materials (EEM). EEM-covered eggshells unexpectedly showed a significant faster water loss than the clean shells. I cannot adequately explain this, as ‘wicking’ of water through an oily layer is counter intuitive. This issue requires further testing.

An aspect to keep in mind was that the tests were carried out at an air pressure of 80.7 kPa at an altitude of 1300 m above sea level. The normal air pressure at sea level where the penguins nest is 101 kPa. Differences in pressure may affect the amounts of water loss, but cannot explain lack of effect of eggshell thickness on water loss.

Although this study started off with a concern raised by thinner eggshells associated with increase POPs concentrations in the eggs, I could find no significant water loss effect related to differences in eggshell thickness. This finding does not negate that thinner eggs are not a problem since the eggs still need to withstand other forces such as body pressure exerted by the incubating parents. Thinner eggs are also more susceptible to infection.

The effect of water loss affected by temperature and humidity through African Penguin eggshells on their population size remains to be determined. However, I showed that increased temperatures are likely to increase water loss rates, thereby placing metabolic stress on the developing embryo. The compensatory abilities of the embryo might be exceeded at such higher temperatures. Climate change through increasing summer temperatures therefore, will add pressure on African Penguins though accelerated water loss.

Key words: African Penguin, Eggshell thickness, Climate change, Water loss, Persistent organic pollutants

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BIS	Bird Island
CORDEX	Coordinated regional climate downscaling experiment
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
EEM	Eggshell extraneous matter
IPCC	Intergovernmental panel on climate change
IUCN	International Union for Conservation of Nature
LRT	Long-range transport
%CVs	Percentage coefficient of variations
NaOH	Sodium hydroxide
PCB	Polychlorinated biphenyl
POPs	Persistent organic pollutants
RIS	Robben Island
SAM	Shell accessory material
SEM	Scanning electron microscope

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CHAPTER 1: INTRODUCTION AND LITERATURE STUDY

1. INTRODUCTION

When reviewing the Earth's climate trend over time, it is clear that it is consistently changing. However, in the past decades, there has been a surprising acceleration in how quickly the Earth's climate is changing. An assessment by the Intergovernmental Panel on Climate Change (IPCC) concluded that due to an increase of anthropogenically driven greenhouse gasses, the Earth is warming dramatically (Colenbrander & Bavinck 2017; Lwasa et al. 2018). Various assessments from the IPCC confirmed that the air temperature would increase 1.8 – 4.0 °C closer to the end of the 21st century if greenhouse gas emissions continue in its current trend (Colenbrander & Bavinck 2017). Climate change will result in extreme environmental events including temperature shifts, rising sea levels, storms, and droughts.

1.1 Climate change and global warming

Firstly, it is important to distinguish between climate change and global warming. Global warming refers to an increase in greenhouse gas concentrations in the air that in all likelihood will lead to an increase in global temperature. Climate change, on the other hand, is the already increased changes in climate, for example wind, humidity, and temperature (Anastasiadis 2005). Due to greenhouse gas emissions from burning fossil fuels described as global warming, it is having negative influences on biodiversity (Lu et al. 2018).

Impact assessments of climate change usually make use of global climate models (GCMs) (Nissan & Conway 2018). In Southern Africa, climate changes are likely to result in decreased precipitation associated with an increase in drier days, thus leading to higher risks of heatwaves, as projected by the Coordinated Regional Climate Downscaling Experiment (CORDEX). Cape Town is now experiencing various changes including a warming climate accompanied by shifting wind regimes (Colenbrander & Bavinck 2017), and droughts. When an increase of 1.5 – 2°C occurs in Cape Town, there will be an expected 20% decrease in rainfall (Weber et al. 2018).

When sea surface and air temperatures rise by about 2°C, it is projected that sea levels will rise between 3 – 5 m, globally (Lwasa et al. 2018). The area along the coast of West Africa is especially vulnerable to climate change and its impacts, because of its low elevation (Chandrashekeran et al. 2017). Not only will the coastal areas exposed be exposed to saltwater intrusion due to sea level rise, affecting adjacent freshwater resources, but there will also be increased coastal and beach erosion. When these changes occur, it will have drastic impacts on coastal sea birds, particularly the African Penguin *Spheniscus demersus* that use the shore for nesting and depends on certain ambient environmental conditions for egg incubation, hatching, and raising chicks.

1.2 Impacts of climate change on seabirds

When sea surface temperature rises due to climate change, scientists found new evidence that there is a 15% decrease in the Atlantic Ocean's circulation (Caesar 2018). This may well affect penguins in general and the African penguin in particular as the distance African Penguins will have to travel farther from their breeding sites in search of food such as euphausiid organisms (Thompson & Hamer 2000) and fish such as anchovies (*Engraulis capensis*) and sardines (*Sardinops sagax*) (Alheit et al. 2012). When environmental conditions changes, such as with increased temperatures and movement of their prey, the behaviour and of the African Penguin will be negatively affected (Nadal et al. 2015).

It is expected that seabirds breeding at low latitudes like the African Penguin will experience warming at a lower degree than birds at a higher latitudes (Crawford et al. 2010). However, they are still adversely affected by climate change through an increase in the severity of El Niño Southern Oscillations frequencies (Thompson & Hamer 2000). These occurrences usually lead to a high mortality rate that also includes breeding failures (La Sorte & Fink 2017).

1.3 Impact of pollutants on seabirds

Climate change and exposure to persistent organic pollutant (POPs), metals, and other pollutants are now regarded as one of the most concerning anthropogenic threats to biodiversity, including birds (Newman 2015; Jenouvrier et al. 2017). There are only a few studies on the combined effects of climate change and pollution in coastal ecosystems since this combined threat is a recently realised concern.

Pollution can occur via over-enrichment of nutrients, high metal concentrations, POPs, and combinations thereof (Nadal et al. 2015). We should especially be worried about the ability of birds to adapt to environmental changes due to climate change. Several organochlorine compounds, for example DDT, pose a threat to the endocrine systems of birds (Baldassin et al. 2016). Various endocrine systems are essential for producing effective physiological and behavioural responses to

environmental stress (Morita 2016). POPs were used and produced worldwide in various processes such as agriculture and malaria control, thereby polluting water, air, and biota in all types of environments (Brogan et al. 2017). Previous studies gave us the understanding of how POPs move in the environment via an air-sea exchange system and the impacts it may have on the environment (Mwangi et al. 2016). One major impact is the accumulation of POPs in ocean-living organisms resulting in bioaccumulation in predators like fish-eating birds including the African Penguin (Mwangi et al. 2016). When there is high accumulation of POPs occurring in birds it may have adverse effects (Luek et al. 2017).

1.4 Impact of anthropogenic pressures on seabirds

Monitoring of fish-eating birds is commonly used to determine the potential risk of secondary poisoning (from source, to sea, to fish, to birds) by POPs (Sakellarides et al. 2006). This is especially true in pre-fledging seabirds because concentrations in their tissues not only to reflect possible health condition, but also their local environment. The ocean plays an important role in the biogeochemical cycle of trace elements and numerous habitats are highly affected. Anthropogenic events and environmental pressures affect the wellbeing of seabirds and bioaccumulation occur in these top predators can result in relatively high concentrations (Falkowska et al. 2016). Changes in the status of pollution in the coastal environments result in changes in population numbers, breeding success and survival.

There are numerous anthropogenic pressures that affect seabirds living on or near the coasts, for instance; POPs pollution, heavy metals, oils, depletion of fish stocks, and plastics (Thompson & Hamer, 2000). I briefly discuss some of them.

Heavy metals including mercury and cadmium have been found in seabirds. The concentration levels are even higher in seabirds than levels recorded in terrestrial birds. For example, Rockhopper Penguins *Eudyptes chrysocome* had exceptionally high cadmium concentrations in their kidney tissue (Thompson & Hamer 2000), and organohalogenated compounds in their eggs (van den Steen et al. 2011).

Pelagic seabirds have the ability to detoxify the organic methyl mercury they have ingested from their prey and thus store less of the inorganic form. This is especially important for large, long-lived, and slow moulting seabirds like penguins which are able to accumulate large amounts of mercury during their life with little possibility of excretion through their feathers following moult (Dauwe et al. 2003).

Oil is another problem for penguins because it floats on the surface and comes directly into contact with the birds while swimming (Thompson & Hamer 2000). When oil gets in the penguin's plumage, it affects their thermal insulation and buoyancy. Even penguin eggs can be affected by oil when they come in contact and may lead to embryo mortality (Thompson & Hamer 2000).

Ever since the use of plastic began, it had a considerable effect on the environment and its effects are only increasing. Plastics such as bottles, bags, and fishing lines

can affect seabirds in a number of ways especially when entanglement and ingestion occurs (Thompson & Hamer 2000).

Lastly, depletion of prey stocks is a serious issue that is inflicted by fishing and climate change (Trathan et al. 2014). The Benguela Current system of southern Africa is also affected by climate change, and has an impact on seabirds. These impacts are mostly felt by species with surface-feeding habits, restricted foraging ranges, and limited energy and time for foraging like the African penguin (Jenni & Kéry 2003).

1.5 Bioaccumulation of pollutants

When pollutants such as POPs reach the sea through different ways like atmospheric transportation, deposition, and surface runoff, the fish are exposed. This leads to bio-concentration (directly from water) and biomagnification of these compounds to the next link in the food web. These two processes combined is called bioaccumulation (Newman 2015). As penguins consume food (mostly fish, crustaceans, and squid) from various trophic levels, they make it possible to measure the contamination concentration around the coastline (Carravieri et al. 2017). This is called biomonitoring (Newman 2015).

Coastal marine ecosystems consist of a variety of organisms and systems supporting many marine and coastal bird species. Marine birds depend to a major extent on the ocean and are therefore excellent bio-indicators of POPs. POPs have become a real concern in the last few decades because they persist in the environment for long periods due to their ability to resists degradation (Innocentia et al. 2019). POPs pose a threat to all animal and human life via bioaccumulation through the food web to levels that may elicit adverse effects. When high concentrations of POPs are taken up by animals, it may cause immunotoxicity, neurotoxicity, developmental toxicity, endocrine disruption, mutagenicity, and carcinogenicity (Nadal et al. 2015).

POPs can be found almost everywhere due to their ability to travel long distances via air and ocean currents through a process called long-range transport (LRT). In previous years, these substances were used in large volumes that led to the exposure of birds to a broad mixture of chemicals that has had, and most probably still has, a significant impact (Huber et al. 2015).

As previously discussed, the environmental distribution and biological effects of chemical toxicants have attracted interest (Newman 2015; Jian-bin et al. 2006). The introduced POPs in the environment are influenced either directly or indirectly by environmental factors such as temperature, wind speed, solar radiation, and precipitation; overall, it is being altered by climate change (Booth & Rahn 1990). These alterations enhance the volatilization from primary to secondary sources and movement between soil, atmosphere, sediment, and water. For example, higher rainfall may lead to POPs deposition from the air to the soil. Storms and floods

enhances the movement of POPs in the soil via runoff to aquatic systems, and hence to aquatic organisms (Nadal et al. 2015).

Seabirds have been widely used in monitoring marine pollution because they feed upon a wide range of trophic levels from the littoral to the pelagic zone. Marine fish accumulate contaminants in their body fat. Many species travel long distances, bringing them into contact with regions that differ in ambient concentrations and compositions of pollutants. Seabirds including the African Penguin feed upon these fish and hence bioaccumulation occurs making them sensitive to environmental contamination and excellent for studies of long-term monitoring (Hong et al.2014).

African Penguins can reflect the contamination pattern of an area because they have the following characteristics: i) They have a relatively long lifespan and accumulate contaminants through their food which is then transferred to their eggs; ii) Biomagnification occurs due to their position in the food web; and iii) They are endemic to the south-west coast of Africa reflecting the contamination distributed in that specific area (Zapata, et al.2018).

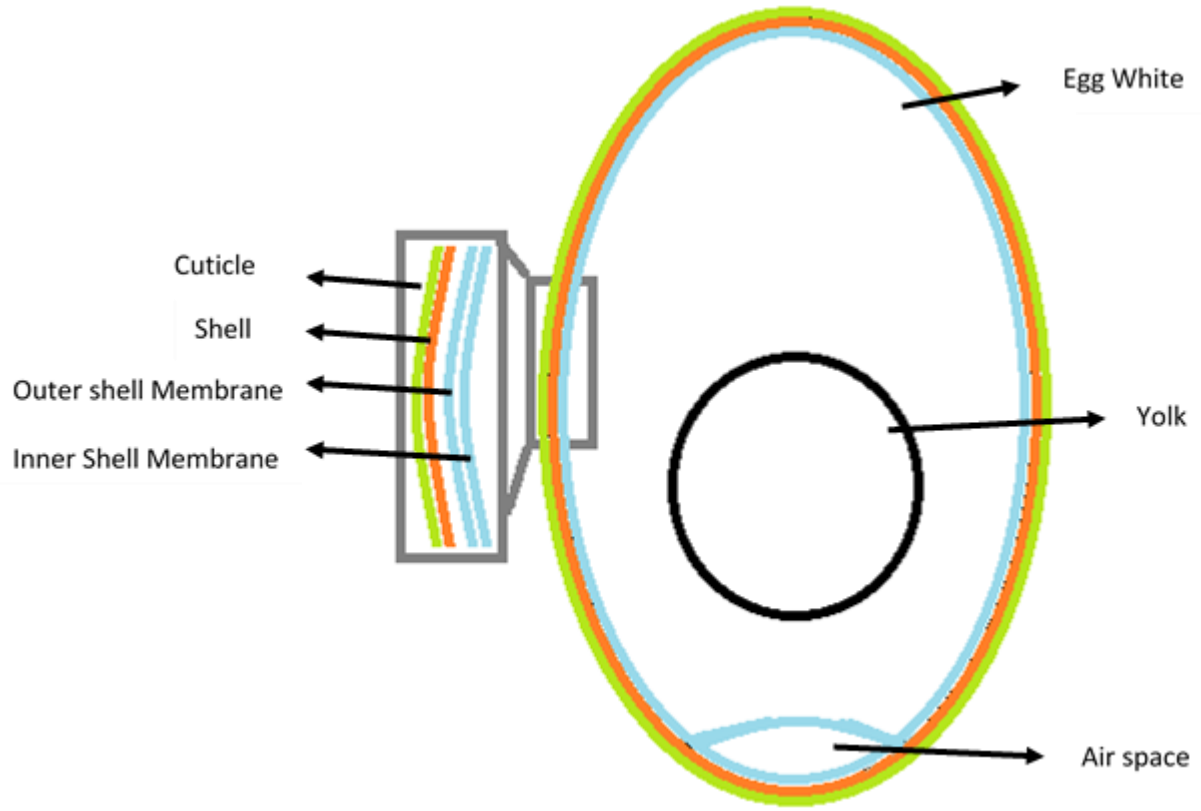


Figure 1.1: Structure of an egg with its multi-layered eggshell. See Figure 1.2 for more detail. Redrawn from Hincke et al. (2012).

1.6 Structure and functions of a bird eggshell

1.6.1 Eggshell structure

The avian eggshell is a well-ordered structure with various layers (Figure 1.1, 1.2) composed of 96% calcium carbonate and other minerals within an organic matrix (Kern et al. 1992; Mortola 2009; Maurer et al. 2012; Hałupka & Orłowsk 2015). The embryo is protected from the environment and physical pressure by the evolutionary innovation of the eggshell, which also has important regulation functions. For example, the eggshell regulates the loss of water, facilitates the absorbance and use of calcium during embryo growth, and enables gas and heat exchange with the surrounding environment (Paganelli 1980; Mortola 2009; Hałupka & Orłowsk 2015). All of this is made possible through highly organised layers (Figures 1.1 and 1.2).

The eggshell is made up of four distinct layers, starting with the innermost mammillary zone, followed by the palisade, the vertical crystal layer (these three layers form the “true shell”), and lastly the cuticle (Figure 1.2). Therefore, the eggshell is composed of different barriers to enable it to overcome constraints imposed by the environment. The outermost layer, namely the cuticle, is a non-calcified layer but it has many functions that impart colour, protection from microbial infection, exposure to solar radiation, and much more (Peebles et al. 1987). Eggs are laid in all types of habitats, even with extreme humidities and temperatures, which can be challenging to embryonic development (D’Alba et al. 2017).

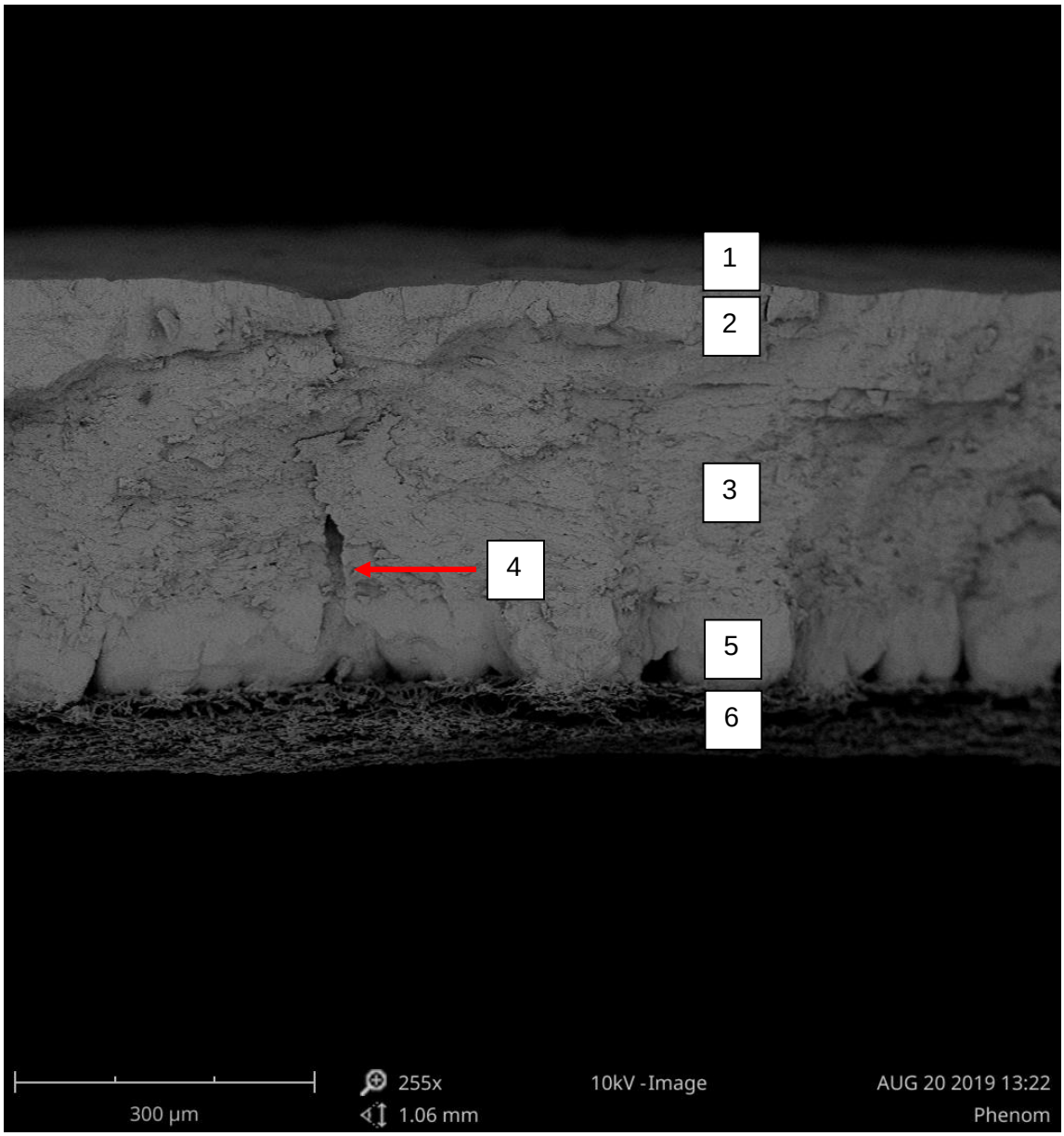


Figure 1.2: Electron micrograph of a lateral view of a cross section of an African Penguin (*Spheniscus demersus*) eggshell using a scanning electron microscope indicating the layered structures: 1 Cuticle; 2 Vertical crystal layer; 3 Palisade layer; 4 Pore canal; 5 Mammillary zone; 6 Membrane.

A penguin eggshell (as is the case for any bird egg that is incubated) must be strong enough to hold the mass of its incubating parent. Actually, the penguin eggshell forms a relatively high percentage of the total egg mass when compared with other birds (Williams 1995). The strength of the shell is directly proportionate with shell thickness, thicker eggs being stronger (Solomon 2010). A previous study found that Yellow-Eyed Penguins *Megadyptes antipodes* had an increase in eggshell thickness associated with an increase in female age, most likely because the more experienced females were more efficient at hunting and catching fish rich in calcium content during the period in egg formation (Massaro & Davis, 2004).

Eggshell extraneous matter (EEM) is the term describing the extraneous layers found on the outer surface of the calcitic shell. EEM is an important contributor that determines the resistance of water loss (Board & Scott 1980). When effective, the EEM controls gas diffusion (Board & Scott 1980). When the EEM is removed, there may be a significant increase in water loss through the penguin eggshells. This is a problem especially for eggshells that also have a high water loss due to other factors like thinner eggshells and external environmental factors. Therefore, these vulnerable eggs need to be incubated in environments with high humidities, but unfortunately, this is often not the case due to climate change (Deeming 2011). Consequently, different bird species have adapted their eggshells' shape, size, and structure to different environments (Stein & Badyaev 2011).

1.6.2 Quality of bird eggshells

The quality of eggshells before it is laid is affected by several factors. Eggshell thickness, for example, is affected by the period it spent inside the uterus where shell formation occurs (de Araújo et al. 2017). Eggs that spend a longer time in the uterus will have thicker eggshells when compared to other eggs that have a shorter eggshell formation period. Warm weather is yet another factor affecting eggshell quality. Increased breathing by the hen (known as panting in domestic chickens) is used for cooling (Barott, 1937). This causes a lowering of CO_2 levels in the blood of the bird and the occurrence of respiratory alkalosis (Ruzal et al. 2011). When this happens, the calcium available in the blood for eggshell formation decreases leading to softer eggshells.

Some eggshells have the ability to reflect UV from the sun; this property is usually associated with pigmentation (Cassey et al. 2012). The reflection decreases the amount of light that enters through the eggshell and therefore prevents embryonic overheating (D'Alba et al. 2017). However, the African Penguin eggshell has very little to no pigmentation; they have light-coloured shells (Figure 2.2) and therefore do not have the ability to reflect UV to the extent that pigmented shells would be able to.

Some females need to ingest calcium-rich items like mollusc shells before egg-laying (Boersma et al. 2014; Vanstreels et al. 2019). Penguin eggs have a high proportion of shell mass among other seabirds because they lay their eggs on hard surfaces with little nesting material and incubate them for long periods. Therefore, calcium ingestion is important to the female even after egg-laying to replenish depleted calcium (Boersma et al. 2014).

When embryonic development is completed, more than 80% of the required calcium is derived from the eggshell (Österström et al. 2013). Thus, thick calcium-rich eggshells are important as only 20% or less of the total somatic calcium is derived from the yolk. The calcium in the eggshell is mobilised from the mammillary tips (Figure 1.2). The equator around the eggshell is thinner at the time of hatching due to calcium mobilisation and results in crater-like remains, whereas the mammillary tips at the blunt end show little change probably because of the intervening air

space. Calcium is vital for embryo skeletal development. If there are an insufficient number of mammillary tips, the skeleton will be less ossified that will lead to other developmental complication (Österström et al. 2013).

The eggshell structures of coastal birds can easily be differentiated from the eggshell structures of inland birds. Due to higher humidities prevalent at coastal regions, the eggshell structures are usually larger, thicker and have higher pore densities (Stein & Badyaev, 2011). Even though the African Penguin can maintain the temperature of their eggs during incubation, fluctuations in ambient humidity during incubation cannot be compensated for (Wilson & Gremillet 1996). This implies that ambient humidity could be one of the reasons for differentiated eggshell structure (Kern & Cowie 2000; Deeming 2002). Thus, optimal eggshell structures are important for successful embryo and chick hatching.

The amount of water loss across an eggshell is in part also determined by the number of pores, the length of the pores (eggshell thickness), and total pore area. Longer pores, for example have less water loss than shorter pores; therefore, the number of pores should be in proportion to eggshell thickness for successful embryonic growth (Jaeckle et al. 2012)**.** This implies that eggs of bird species nesting in warm and humid environments should have a greater pore area when longer pores are present in the eggshell in comparison with similar sized eggs of bird species breeding in cold and drier environments, allowing optimal water loss by pore area compensation (Stein & Badyaev 2011).

1.7 Environmental contamination and bird eggshell thinning

The fish and euphausiids that penguins feed upon, although valuable in sources of vitamins and fatty acids such as omega-3, also contain harmful endocrine-disrupting compounds. This includes the previously mentioned organochlorine compounds (pesticides and fungicides). Larger eggs of piscivorous species seem to have higher concentrations of POPs and trace elements as was the case for African Penguin eggs (Clatterbuck et al. 2018).

DDT has been used for insect control as a pesticide, but it was banned in South Africa in 1974. Yet, there is limited use of DDT allowed to fight malaria in the north-eastern regions of South Africa until 1995, and resumed in 2000 following an increase in the incidence of malaria in the north-western parts of the country (Ryan et al. 2012; Mo et al. 2018).

When seabirds ingest these POPs, it may result in endocrine disruption leading to abnormal breeding behaviour, poor breeding success, and reduced fertility amongst a number of other effects (Baldassin et al. 2016). POPs are able to act or mimic endocrine hormones and alter or modify endogenous hormone functions. In several studies, it was found that POPs have a significant effect on reproduction hormones (Sakellarides et al. 2006). The effects of toxicants may also manifest when the

penguins are involved in activities that have high energy costs like breeding due to lipid mobilization and its redistribution between tissues (Baldassin et al.2016).

Not only are disturbances of reproductive hormones the cause of reproductive failure but there is also a long-known link between DDT and its derivative DDE and eggshell thinning (Lundholm 1997). Eggshell thinning raised concern in the face of declining bird populations. The degree of thinning varied in response to DDE and PCB concentrations (Bouwman et al. 2015; Falkowska et al. 2016). According to the study by Bouwman et al. (2015), mean DDT concentrations in the African Penguin eggs remained the same over the past 30 years, but PCB concentrations declined four-fold. On Bird Island (BIS) near Port Elizabeth, the difference in eggshell thickness (thinnest to thickest) was 38%, and it was 40% at Robben Island (RIS) near Cape Town. The thinner African Penguin eggshells associated with increased organic pollutants (especially polychlorinated biphenyls; PCBs) which possibly affect population numbers was the main reason for this study. The same eggshells used by Bouwman et al. (2015) were used in the present study.

1.8 Water loss through bird eggshells

The avian eggshell not only functions to prevent breakage from pressure, external harm, or provide nutrients to the embryo, but it also regulates gas exchange across the shell, between the external and internal environments (Paganelli 1980; Mortola 2009; Hałupka & Orłowsk 2015). From the moment the egg is laid, water is lost as vapour from the inside to the outside of the shell. The egg therefore becomes lighter with time. The eggshell structure, including its thickness, regulates the amounts of water loss. At the end of the incubation period, when the embryo is ready to hatch, ideally there would have been an optimum weight loss (Ar & Rahn 1980; Carey 1986). Optimum weight losses seem to differ between species; it is 11.5% for Turkey eggs (Hullet et al. 1987), 14% for tern eggs (Rahn et al. 1976), 12.5% for chicken eggs (Davis & Ackerman 1987), 20% for the Eastern Screech Owl *Otus asio naevius* (Ar & Rahn 1980), and 5.2% for African Penguin eggs (Yom-Tov et al. 1986). The transportation of water across the shell to the external shell environment (also called evapotranspiration) will result in concomitant amounts of other gasses such as oxygen to replace it.

Water loss is affected by ambient humidity because humidity outside the egg influences the water pressure gradient inside the egg and the air surrounding the egg. The greater the water pressure deficit, the greater the water loss from the egg to the atmosphere (Mortola 2009). During high temperatures, the percentage of relative humidity outside the egg will decrease meaning that there would likely be an increase in water diffusion through the eggshell to the outside air. Regardless of temperature, when the humidity is too high outside the egg, water loss through the eggshell may not be sufficient. Insufficient water loss of the egg when pipping occurs may lead to mortality, because full inflation of the lungs inside the egg's air space (Figure 1.1) will not be possible for the chick (Noiva et al. 2014). For this reason,

climate change does not only affect hatching success by increased temperatures but by changes in humidity (IPCC 2013). Intermittently high temperature spikes caused by global warming and climate change can affect embryonic development and hatching (Griffith et al. 2016; Ton & Martin 2017). Eggs can experience high temperatures in natural conditions. However, although eggs from the African Penguin can experience nest temperatures of up to 40˚C (Lei et al. 2014), it should be kept in mind that equator eggshell thickness differences occur at up to 40% between the thickest and thinnest eggshell (Bouwman et al. 2015).

There is little information about the actual water loss through eggshells during different ambient temperature and humidity treatments in relation to eggshell thickness. Previous studies looked at mass loss for whole eggs during incubation (Nakage et al. 2003; Deeming 2011).

1.9 The African Penguin

The subject of my study is the African Penguin *Spheniscus demersus*.

Spheniscus, the genus name, is derived from Greek, which means wedge referring to their streamlined body, while *demersus,* the species name, is derived from Latin, which means sinking, referring to their diving behaviour (Hockey et al. 2005).

The African Penguin is endemic to the coast of southern Africa. The African Penguin can easily be identified by their pink eyebrow, black dorsal part, and spotted white belly plumage. Their white belly has one black stripe curving across the top of the chest and downward toward the legs. Every African Penguin individual has a unique pattern of black spots on their white belly area that can be a way to identify individuals (Figure 1.3). Juveniles can be distinguished from the adults due to their blue-grey plumage.

The African Penguin has donkey-like braying noises, hence their common name “Jackass”. These flightless birds use their wings as flippers for efficient swimming accompanied by their streamlined bodies and waterproof feathers. They are highly specialised swimmers with a swimming speed reaching up to 20 km/h and can dive down to 130 m.

Adult African Penguins weigh between 2.2-3.5 kg and can reach 60-70 cm in height. When foraging, the African Penguin prey on small pelagic fish and euphausiids. They can travel very long distances especially during non-breeding times in search of food. However, foraging does not occur during night.

African Penguins are able to breed throughout the year, but their peak breeding season tends to be during March to May in South Africa, and November to December in Namibia. They usually lay between one and two eggs and incubate the eggs 38-41 days. Both parents are responsible for incubation; thus, when one parent incubates the egg the other will head out to forage (Hockey et al. 2005).

The African Penguin has been experiencing rapid population declines during the past century. There used to be around one million breeding pairs in the 1920s. However, they have declined to about 52 000 adults in 2012 (Crawford, et al.1995). It is estimated that they have experienced a 95% decline from 2001 to 2011. This resulted in the bird being classified as Endangered by the International Union for Conservation of Nature (IUCN) Red List (BirdLife International 2012).

There is limited breeding space for the African Penguin, therefore, artificial nests were created and placed on the beach. Due to climate change, there is an increase in temperature and natural burrow nests are exposed to higher than ambient temperatures (Griffin 2005). However, eggs incubated in these artificial nests are exposed to even higher temperatures that can easily reach 40˚C, and they stay warmer than natural nests for longer (Lei et al. 2014). The incubating parents are also exposed these extreme temperatures and are likely to experience heat-stress (Šálek & Zárybnická 2015). When the African Penguin experience overheating, they may leave the eggs unattended for some while to try and cool down (Stein & Badyaev 2011).

Figure 1.3: The African Penguin (*Spheniscus demersus*) showing the characteristic pink eyebrow and diagnostic half-moon across its chest (Sherley 2013).

1.10 Problem statement and predictions

Differences in bird eggshell thicknesses occur due to numerous factors, including thinning due to POPs. Not only does thinning weaken the shell, but also weaker shells combined with elevated ambient temperature and changes in humidities may result in changes in water loss rates from the egg. As such, it is hypothesised that thinner eggshells may loose water more rapidly than would be optimal for suitable embryo development at lower relative humidities and higher temperatures. This may affect hatching success and subsequent chick survival. A previous study on African Penguin eggs found mean eggshell thinning of 38% and 40% from two different islands, associated with pollutants (especially PCBs) in the egg contents, raising serious concerns about the desiccant effects climate change may have on hatching success and subsequent chick survival (Bouwman et al. 2015; Veldsman et al. 2020).

I developed and tested a laboratory method that measured water loss through different thicknesses of eggshells (chicken eggs) at controlled temperatures and humidities to ascertain the combined effects of climate change (represented by varying temperature and humidity) and changes to eggshell thickness on bird reproduction (Section 3.8 and 4.8) (Veldsman et al. 2020). It was found that eggs at low humidity lost more water than at higher humidity, and more water was lost at higher than lower temperatures. It is expected to be the same for penguin eggshells. With this method, I will test various temperature and humidity scenarios on different eggshell thicknesses of the African Penguin and relate the findings to possible threats to the African Penguin.

Aim:

The aim of this study was to investigate the possible effects of climate change on water loss through eggshells of different thicknesses and the threats it may pose to the African Penguin.

Objectives:

- To determine water loss via evapotranspiration through different thicknesses of eggshell at different temperatures and humidities.
- To determine the effects of pore density on water loss.
- To determine the effect of eggshell extraneous materials on water loss.
- Determine if there is any difference in water loss between eggshells from two different African Penguin colonies.
- To relate the findings of this study to the already endangered African Penguin.

Predictions:

- The rate of water loss is influenced by the thickness of the eggshell.
- The amount and rate of water loss is related to temperature and humidity.
- Water loss is affected by the pore density of the eggshell.
- Water loss is affected by eggshell extraneous materials.
- Thinner eggshells under changing climate conditions may affect reproductive outcomes.

"Uphill struggle gets you to dry ground"
-Hindrik Bouwman

CHAPTER 2: MATERIALS AND METHODS

2.1 Egg collection

Twenty freshly laid and incubated African Penguin eggs were collected between December 2011 and January 2012. Ten eggs were collected from Robben Island near Cape Town, and ten from Bird Island near Port Elizabeth (Figure 2.1). The project and collection was approved by the animal ethics committee of the North-West University (NWU) (NWU-00055-07-S3) in the year 2011. The ethical approval for this study was given the number NWU-01945-19-A9 in 2020. Permits for collections were obtained from the relevant provincial authorities. The eggs were opened and emptied for a previous study to ascertain POPs concentrations (Bouwman et al. 2015). The eggshells were washed thoroughly with distilled water and left to air-dry. The eggshells were then stored in individual cardboard boxes in a freezer, and used in 2019. Storing of eggshells during approximately 7 years did not affect the eggshell composition or thickness.

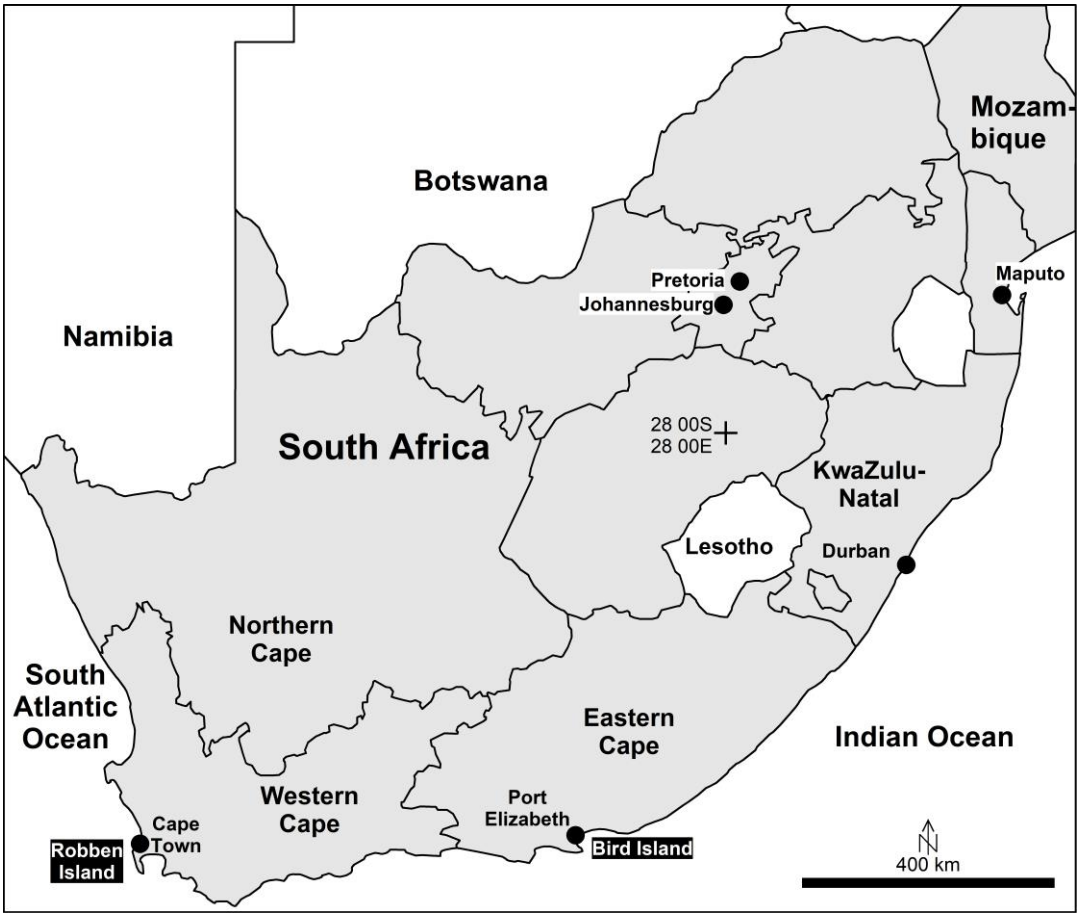


Figure 2.1: Map of South Africa indication the two islands were the eggs were collected (Bouwman et al. 2015).

2.2 Eggshell preparation

I elected to work on eggshell samples cut from the equator region of each egg because multiple such samples can be taken from the same egg (Figure 2.2). This would not be possible if samples from the distal ends were used. Three eggshell fragments from the equator (approximately 2 x 2 cm) from each egg were cut into squares using a Dremel 4000 rotary tool with a 25 mm steel Mini Blade wheel (Figure 2.3). This method produced 60 fragments. All eggshell fragments were measured for thickness at its centre with a digital calliper (Kroeplin B2R20S) accurate to 0.01 mm (http://www.kroeplin.com) (Figure 2.4). The membranes have been removed during sample preparation for the study by Bouwman et al. (2015). Each fragment was measured three times, and the mean was determined. Therefore, the pore length is also obtained for each fragment, as thickness = pore length.

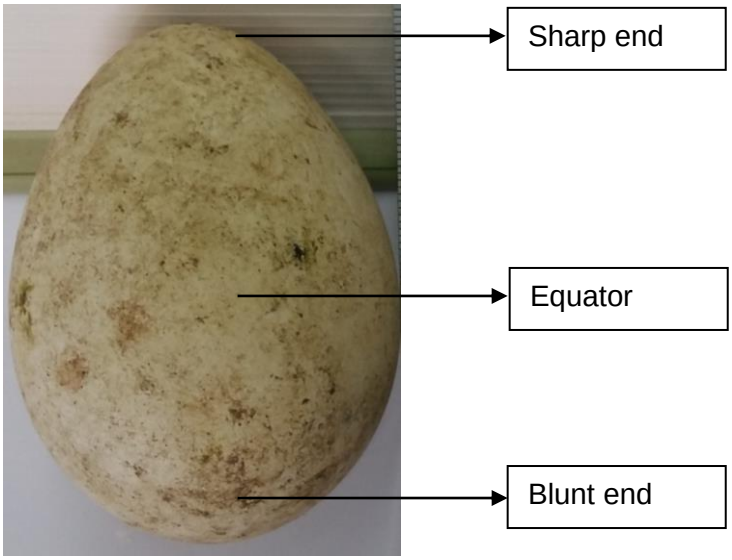


Figure 2.2: African Penguin egg indicating the various regions. Also note the eggshell extraneous matter (EEM), the oily debris covering an otherwise almost white shell.

Figure 2.3: The Dremel 4000 rotary tool with a 25 mm steel Mini Blade wheel that was used to cut the eggshells into square fragments.

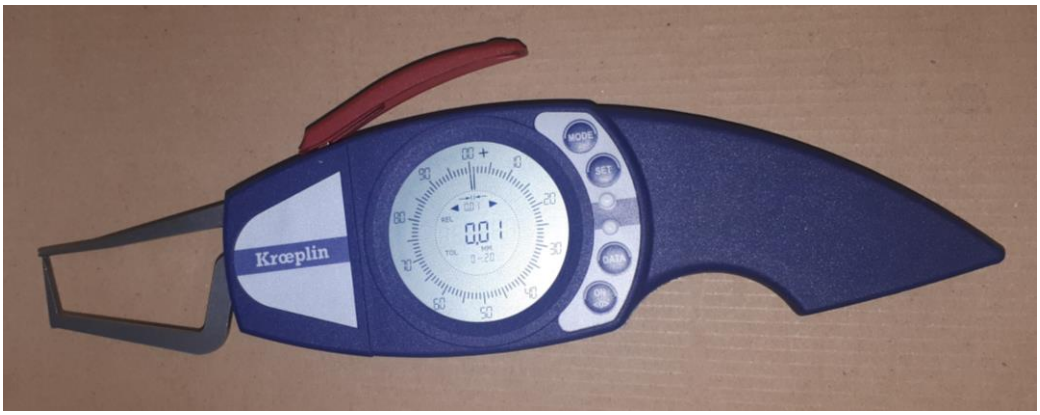


Figure 2.4: The digital calliper (Kroeplin B2R20S) that was used to measure eggshell thickness.

2.3 Eggshell fragment and test unit preparation

Eggshell fragments with the inside facing down onto the vial cap were glued with wood glue (cold glue) onto the outside rim of a screw cap of a 5 ml chromatography vial with a 9 mm diameter opening in the cap (63.61 mm^2) with no septum on the inside (Figure 2.5). To ensure that the eggshell covered the whole opening, the fragment was pressed down with (extreme) care to prevent any glue from covering the inner part of the shell and cap, or breaking the shell. The cap was screwed (again very carefully) onto the vial, now called a 'test unit' (Figure 2.6). Each test unit was labelled and the glue cured for 24 hours. Each fragment was carefully checked to ensure the fragment adhered securely before the second layer of glue was added on the outside to ensure a tight seal. The glue was left to dry for another 24 hours.

Each of the 60 test units were filled exactly with 4.0 ml of distilled water and then closed with the screw cap containing the eggshell fragment. All the test units were placed in a tray for ease of handling and storage. The trays were purpose made to allow enough space (30 mm to next nearest neighbour) between units to reduce possible neighbour effects from released water vapour (Figure 2.7). The trays with the test units were left to equilibrate for three days. All handling of shell fragments and test units were with nitrile gloves to prevent any transfer of moisture from fingers to the test units. Handling of the test units affected the mass of the test unit as was observed during pilot tests.

Using water instead of egg contents has been shown to have no effect on water loss (Taigen et al. 1978). I also tested this with a few African Penguin eggshells, by comparing chicken egg white and distilled water using the same shell fragments. I found no difference in water loss, so continued with distilled water.

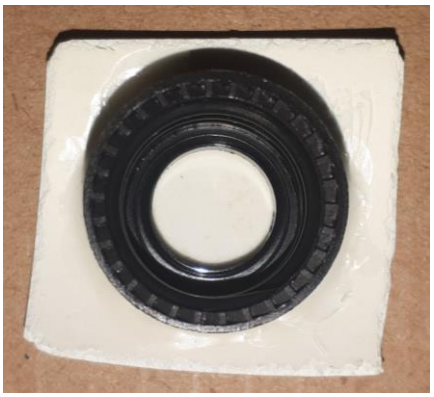


Figure 2.5: Ventral view of the eggshell fragment glued onto a vial cap with a 63.61 mm^2 opening for water loss through the eggshell.

Figure 2.6: View of a test unit with an African Penguin eggshell fragment glued onto the vial cap.

Figure 2.7: Tray holding eggshell test units for treatments.

2.4 Water loss during incubation with different temperatures and humidity treatments

The test units were weighed initially on an electronic scale that is accurate to 0.0001 g. The test units were then placed into a Surehatch 280 egg incubator and hatcher (www.surehatch.co.za) for all experiments (Figure 2.8). The incubator/hatcher has its own built-in temperature controller and fan. Due to the incubators inability to attain temperatures lower than ambient, a mixture of water and glycerol was used, circulating via an aquarium pump inside a plastic bucket filled with water through an external chiller. The glycerol was necessary to prevent the water from freezing because the water reached -5˚C when pumped through the external chiller. An additional external humidifier (Elektra Model 8073, 6-L water capacity, ultrasonic humidifier, humidity controlled, www.sp-africa.com) was needed to blow humidified air into the incubator via plastic tubing right in front of the fan. For settings that required very low to no humidity, the humidifier was turned off and silica gel was inserted to dry out the air by using different quantities, and balancing the quantities to reach the required humidity.

The test units were weighed after three days to ensure minimal interference with the temperature and humidity. All the test units were refilled between different temperature and humidity treatments.

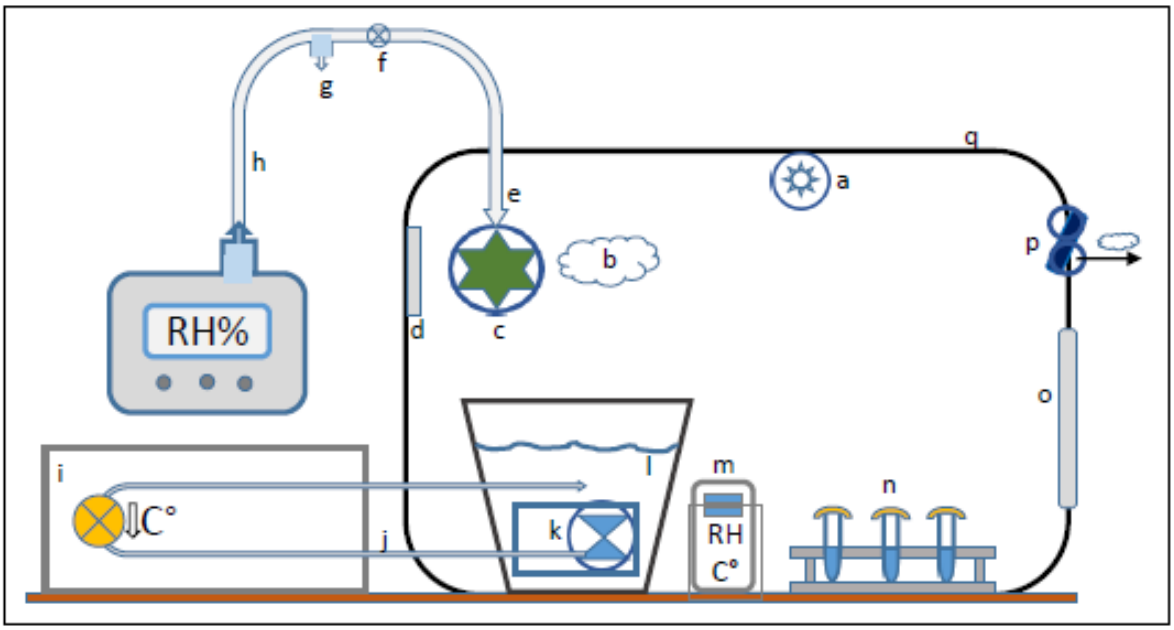


Figure 2.8: Incubator set-up used for different temperature/humidity treatments: (a) lightbulb, (b) humid air blown into incubator, (c) circulating fan, (d) thermostat-controlled heating element, (e) exit of plastic tubing in front of fan, (f) tap, (g) drip trap, (h) commercial humidifier, (i) chiller, (j) flexible plastic tubing, (k) immersed aquarium pump (l) plastic bucket with water and glycerol mixture, (m) electronic temperature and humidity sensor visible through window, (n) eggshell-vials/test units in a custom-made acrylic tray, (o) door with

window, (p) variable humidity regulator, and (q) commercial incubator. Adapted from Veldsman et al. (2020).

The effects of climate change on relative humidity are not homogenous across the world. According to the effects of temperature on humidity as seen in previous temperature-relative humidity occurrences during the past few years at Cape Town (Timeanddate 2019), the relative humidity decreases when temperature increases. Based on IPCC projections, the relative humidity will decrease at least 1-3% between 2016 and 2035 in southern Africa (IPCC 2013). Although the interactions as to why relative humidity decreases with the increase in temperature are not fully understood (IPCC 2013), it will become clear in Figure 3.4.1 what the effects on water through the African Penguin eggshell would be. Due to the decrease in relative humidity, it is expected that more water will evaporate through the eggshells due to the less humid outside air and embryonic stress may occur. All of the temperature and humidity treatments for 18 different relative humidity treatments at six different temperatures (Table 1.1) were selected based on data from climate and weather averages for Cape Town, South Africa during the breeding period of the African Penguin. The 32°C and 37°C treatments and humidities are scenarios of anticipated future climate change.

Table 1.1: The 18 different temperature and humidity treatment scenarios used for African Penguin eggshell incubation. The 32°C and 37°C treatments and humidity combinations are in expectation of possible future scenarios under climate change.

Temperature (˚C)	Relative humidity (%)
12	70, 80, 90
17	60, 70, 80
22	50, 60, 70
27	40, 50, 60
32	30, 40, 50
37	20, 30, 40

2.5 Eggshell staining and pore density determination

Water crosses the eggshell through pores (Figures 2.9 and 2.10). To determine whether water loss is affected by the number of pores, I measured pore density for each egg. Three shell fragments from each eggshell were used to determine pore densities using the technique described by Taylor et al. (2018). These were not the same fragments as used for the test units, but sourced as near to the equator as possible.

Each of the 60 fragments was placed in a separate beaker. Five percent of NaOH solution was added and the fragments were boiled for several minutes to remove

proteinaceous fibres from the inner surface of the shell and the cuticle on the outer surface (Peebles 1987). Following which, they were rinsed in water and left to dry at room temperature. The shells were immersed for a few seconds in nitric acid to enlarge the pores for staining. By trial and error, the correct etching time was found to achieve the desired enlargement of the pores. The reaction was stopped when placed in distilled water. When the fragments were dry, the samples were stained with an aqueous solution of 1% methylene blue, applied by a dropper, on the inside of the shell and left for two minutes. Due to capillary forces, the pore channels became visible as fine blue dots on the external surface of the eggshell (Figures 2.9 and 2.10).

The fragments were left to dry at room temperature. An area of 100 mm^2 was divided into four 25 mm^2 areas by using a template (Figure 2.10). The pores were counted under a stereomicroscope and the sum of the pores on the four areas was determined. The mean was calculated for the three fragments and considered as the pore density per 1 cm^2 for each specific egg. The average pore density per 1 cm^2 was multiplied by the surface area of the egg resulting in the porosity of the eggs.

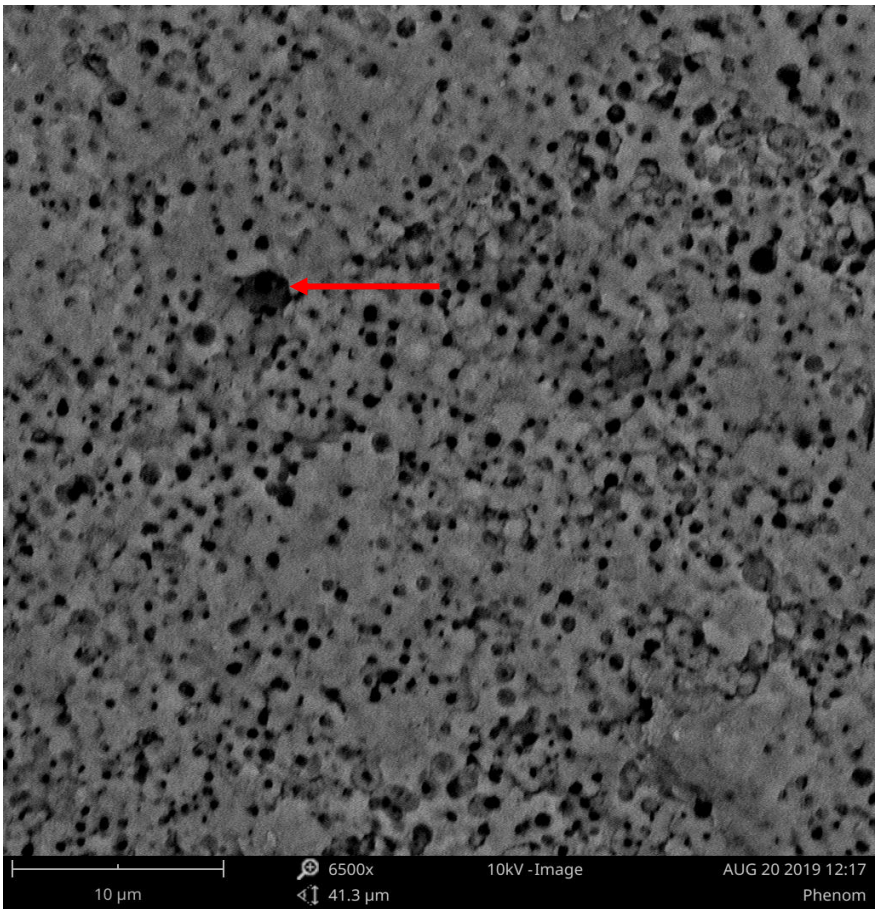


Figure 2.9: Image of the exterior of an African Penguin eggshell using a scanning electron microscope (SEM), arrow indicating a pore.

Each eggshell was marked with a 1 cm^2 red block to ensure only pores inside the 1 cm^2 square were counted (Figure 2.10). Figure 3.5.1 will show how effective this technique was.

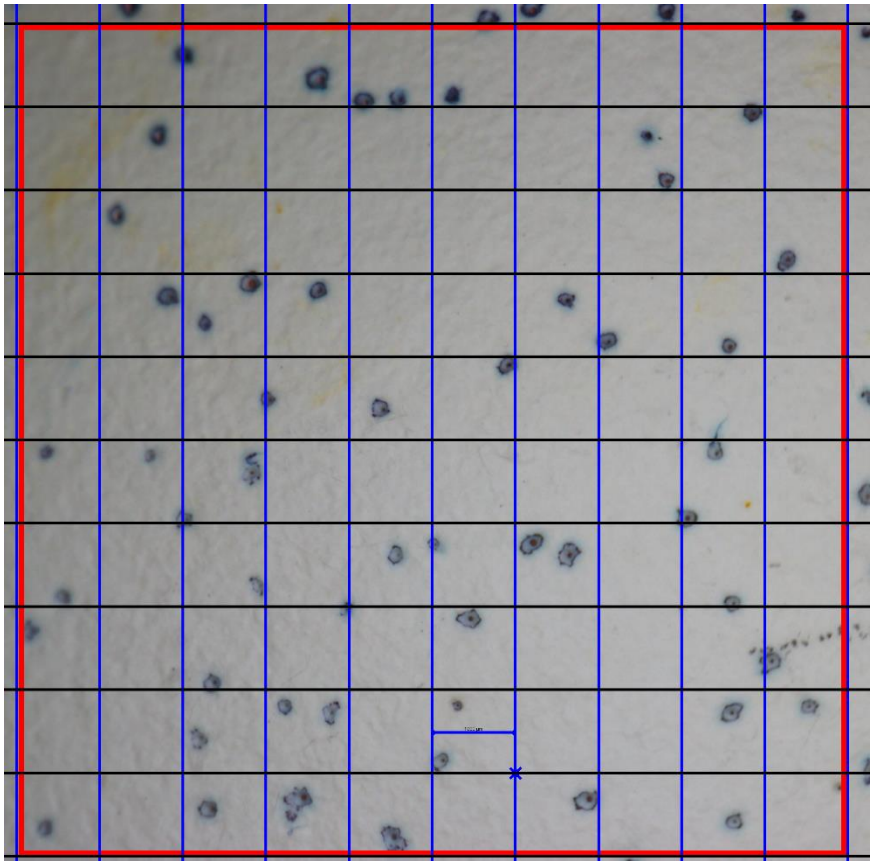


Figure 2.10: Light microscope image of the method used to calculate pore density in 1 cm^2 (red square) of an African Penguin eggshell.

2.6 Comparing water loss between clean and dirty (EEM-covered) eggs

Early on, it became evident that the eggs had different degrees of EEM (Section 1.6.1). Since it is entirely possible that EEM will affect water loss as it covers the pores, I investigated this effect by comparing three fragments of a 'clean' egg and three fragments of a 'dirty' egg (Figure 2.11), in the same way as described in Section 2.2, including the measurement of thickness. The six units were tested in the incubator at 32˚C with 40% relative humidity.

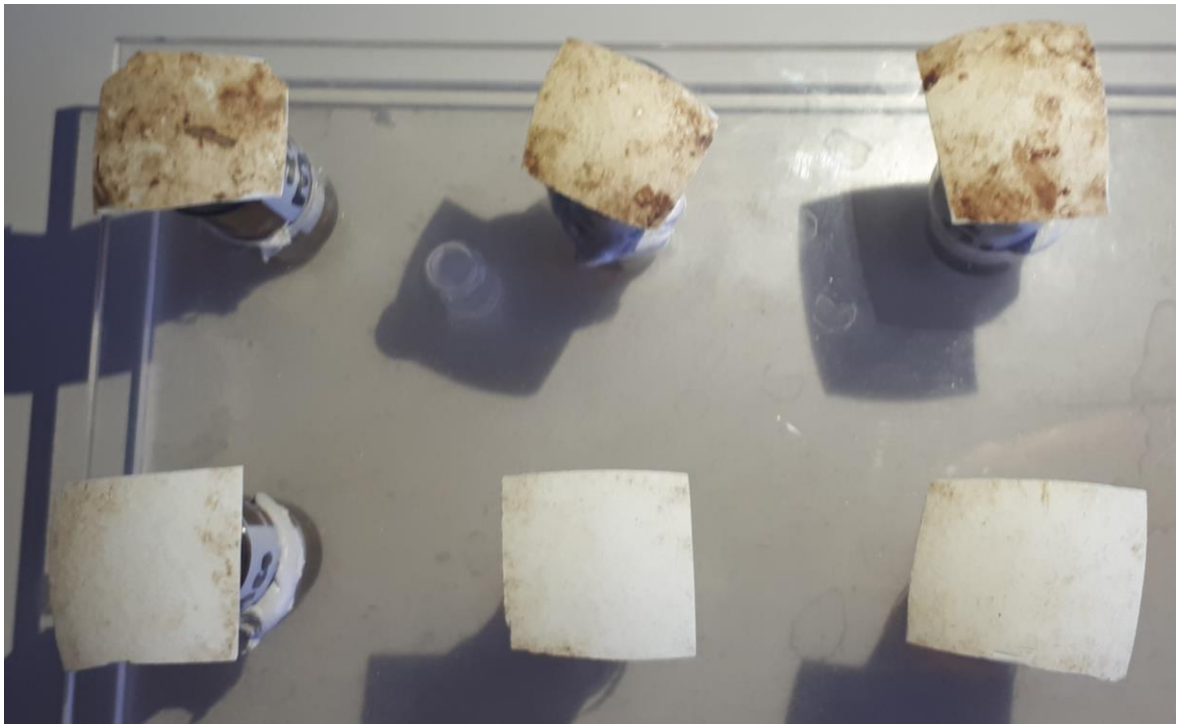


Figure 2.11: Tray holding washed (clean, bottom row) and EEM-covered (dirty, top row) eggshell fragments

2.7 Water loss comparison between chicken and African Penguin eggshells

Additional information was used from data I published (Veldsman 2020). The data contained information on water loss through chicken eggshells by using the same methods described in Section 2.2 and 2.5, however using different temperature humidity treatments. The water loss results received for chicken eggs will be compared to water loss results for the African Penguin.

2.8 Data analysis

Data were analysed using GraphPad Prism 8.0 (www.graphpad.com). Summary statistics included tests for normal distributions using D'Agostino & Pearson omnibus tests. Outliers were identified using ROUT. This method fits a model to the data least sensitive to outliers, then identifies outliers far enough from the predictive model using the false discovery rate. The Q variable was set at 1%. The outliers were removed from the data.

For almost all comparisons, un-paired, two tailed, non-parametric approaches were used. For comparing two sets of data, I used the Mann-Whitney test. For comparing more than two sets of data, I used one-way ANOVA with Tuckey post-tests for multiple comparisons.

For all except one case, I used linear regression to investigate the relationships between the various variables. Apart from testing whether each regression line

differed significantly from zero, I compared regression lines with each other. First, I tested if the slopes were equal (parallel) to each other, providing a p-value, which, if smaller than 0.05, indicates significant differences between the slopes of the regression lines. When the lines were not significantly different from each other in slope, I tested whether the y-intercepts were significantly different. In the text, this is phrased as ‘horizontal separation’ between the regression lines. If the p-value is less than 0.05, it can be concluded that the lines are not identical although still parallel.

The only place I used non-linear regression was when I analysed the %CVs associated with water loss at different temperatures irrespective of the test humidity (Figure 3.4.2). The shape was obviously non-linear. The best-fit non-linear regression line was the ‘sum of two Lorentzian’ lines (basically, inverted Gaussian distributions with wide tails).

“The love for all living creatures is the most noble attribute of man”
-Charles Darwin

CHAPTER 3: RESULTS

3.1 General eggshell characteristics

The results from the African Penguin egg measurements are shown in Table 3.1. There was very little difference in the measurements of each of the three fragments of each shell, so the mean of the three measurements was used to calculate the mean thickness (Table 3.1). There was a 21% eggshell thickness difference between the minimum and maximum mean measurements of the eggshell fragments used in this study. The eggshell thicknesses were also normally distributed with a standard deviation of 0.031 mm. African Penguin eggshell characteristics from a previous study by Amos & Rahn (1985), were added in Table 3.1. According to their results, they found that the eggshell thickness in 1985 were 13.8 % thicker than what I found in this study for eggs collected in 2011 - 2012. The pore density in their study was considerably lower (32% less) than the pore density for the eggs used in this study.

Table 3.1: African Penguin eggshell morphometric characteristics (n = 20).

	Mean	Min	Max
Length (mm)	73.6	67.4	76.6
Width (mm)	54.5	53.2	56.1
Egg surface area (cm^2)	111.6	105.4	120.6
Volume (cm^3)	108.4	100.3	121.5
Thickness (mm) this study	0.50	0.44	0.56
Thickness (mm) (Amos & Rahn 1985)	0.58	N/A*	N/A*
Pores (1 cm^2)	54	33	68
Pore density for the whole egg (cm^2)	9447	N/A**	N/A**
Pore density for the whole egg (cm^2) (Amos & Rahn 1985)	6417	N/A**	N/A**

*Minimum and maximum where not calculated

** Minimum and maximum could not be calculated

3.2 Differences between eggs from Robben Island and Bird Island

A comparison between the 10 eggs collected separately at RIS and BIS were made to determine if there was a difference in eggshells thickness (Figure 3.2.1). The thicknesses were not significantly different (Mann-Whitney, unpaired, two-tailed test; p = 0.2663).

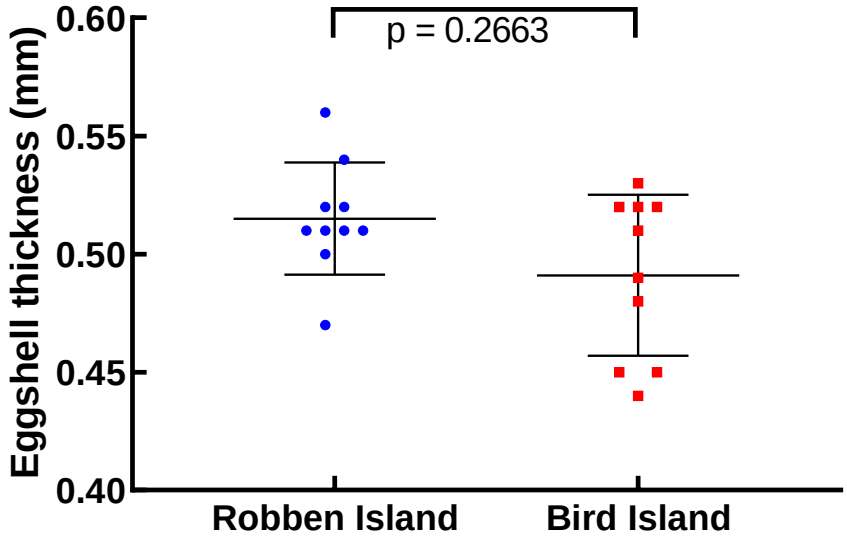


Figure 3.2.1: Scatterplot comparing eggshell thicknesses between the Robben Island and Bird Island. Means and standard deviations are indicated by horizontal bars, as well as the result of the unpaired, two-tailed, Mann-Whitney test.

In Figure 3.2.2, a comparison of pore density was made between the eggs from the two islands to determine if there was a difference between the two breeding sites. There was no difference (Mann-Whitney, unpaired, two-tailed test; p = 0.3051).

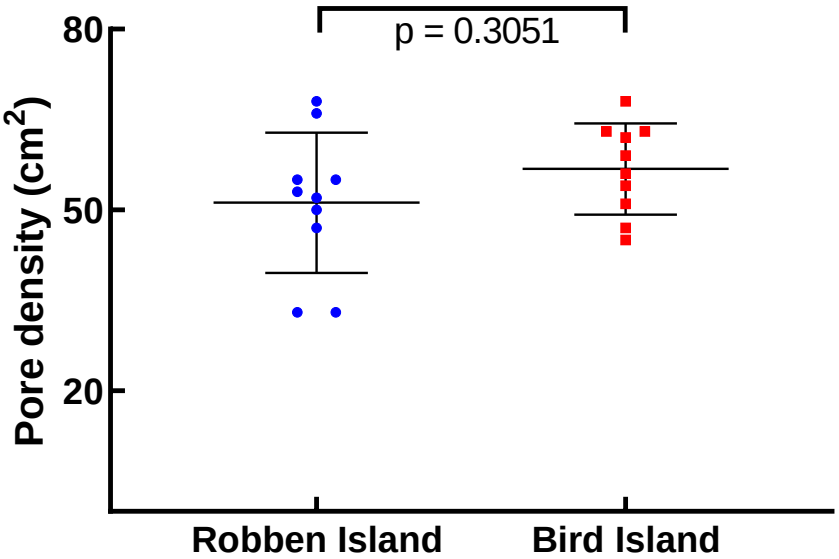


Figure 3.2.2: Scatterplots comparing the mean pore densities in the African Penguin eggshell between the two islands. Means and standard deviations are indicated as horizontal error bars, as well as the results of the unpaired, two-tailed Man-Whitney test

Water loss at different temperatures and relative humidities are presented in Figure. 3.2.3 A-F. Each unit was measured three times over three days for water loss, resulting in 30 measurements per island for each treatment. Supporting data can be found in Table 3.2.

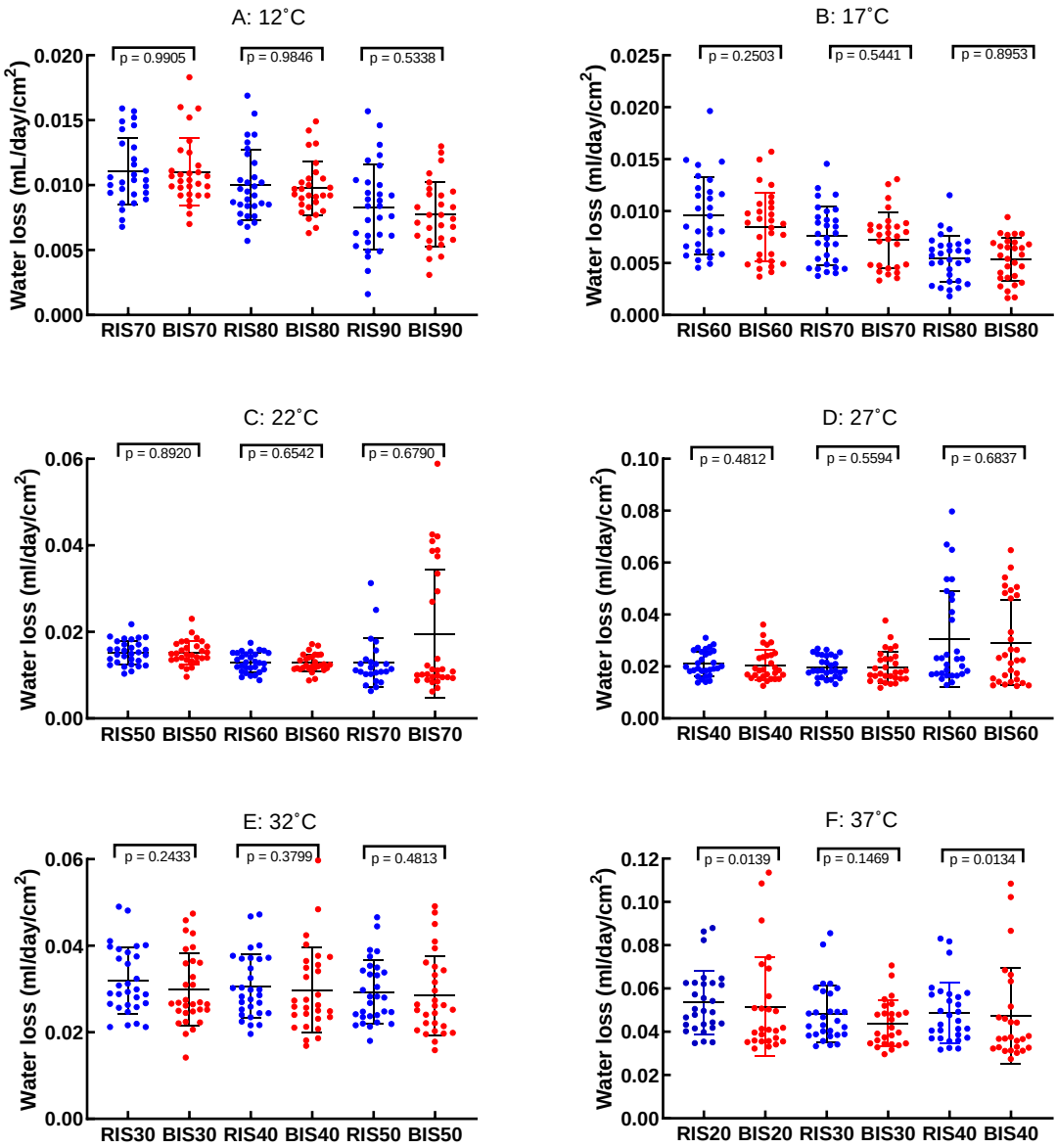


Figure 3.2.3: Scatter plots comparing water loss of the two different islands (Robben Island - RIS and Bird Island - BIS) at the same temperatures for different humidities on each graph. Means and standard deviations are indicated by horizontal bars, as well as the results of the unpaired, two-tailed, Mann-Whitney tests comparing water loss for the two different islands. A: Comparison of water loss at 12˚C across eggshells from two different islands at three different humidities (70%, 80% and 90%). B: Comparison of water loss at 17˚C across eggshells from two different islands at three different humidities (60%, 70% and 90%). C: Comparison of water loss at 22˚C across eggshells from two different islands at three different humidities (50%, 60% and 70%). D: Comparison of water loss at 27˚C across eggshells from two different islands at three different humidities (40%, 50% and 60%). E: Comparison of water loss at 32˚C across eggshells from two different islands at three different humidities (30%, 40% and 50%). F: Comparison of water loss at 37˚C across eggshells from two different islands at three different humidities (20%, 30% and 40%).

Figure 3.2.3 A: There were no significant differences in water loss across the eggshells from eggs from the two different islands at the same temperature and humidity. Further data are provided in Table 3.2. There was a clear trend in decreasing water loss with an increase in relative humidity.

Figure 3.2.3 B: There were no significant differences in water loss across the eggshells from eggs from the two different islands at the same temperature and humidity. Further data are provided in Table 3.2. There was a clear trend in decreasing water loss with an increase in relative humidity.

Figure 3.2.3 C: There were no significant differences in water loss across the eggshells from eggs from the two different islands at the same temperature and humidity. Further data are provided in Table 3.2. There was no clear trend in decreasing water loss with an increase in relative humidity.

Figure 3.2.3 D: There were no significant differences in water loss across the eggshells from eggs from the two different islands at the same temperature and humidity. Further data are provided in Table 3.2. There was no clear trend in decreasing water loss with an increase in relative humidity.

Figure 3.2.3 E: There were no significant differences in water loss across the eggshells from eggs from the two different islands at the same temperature and humidity. Further data are provided in Table 3.2. There was no clear trend in decreasing water loss with an increase in relative humidity.

Figure 3.2.3 F: There were no significant differences in water loss across the eggshells from eggs from the two different islands at the same temperature and 30% humidity, but there were significant differences in water loss at 20% and 40% relative humidity. There was no clear difference in decreasing water loss with an increase in relative humidity.

Based on unpaired, two tailed Mann-Whitney tests, there were no differences in water loss across eggshells of eggs from the two islands for any temperature/humidity treatment, except at the 37°C treatment at 20% and 40% relative humidity.

Conclusion: Based on the comparisons of eggshell thickness, pore density, and water loss of eggs from each island, it can safely be concluded that there are no obstacles in pooling further measurements and comparisons of twenty eggs. I deem the difference as illustrated in Figure 3.2.3 F as not sufficient to keep the two sets of eggshells separate, as this was the treatment at the highest temperature.

Table 3.2: Summary statistics for different temperature and relative humidity (RH%) treatments comparing the water loss for the two islands Robben island and Bird island.

	12˚C						17˚C					
	RIS70	BIS70	RIS80	BIS80	RIS90	BIS90	RIS60	BIS60	RIS70	BIS70	RIS80	BIS80
Minimum	0.0068	0.0070	0.0057	0.0063	0.0016	0.0031	0.0046	0.0037	0.0038	0.0033	0.0018	0.0016
Maximum	0.016	0.021	0.017	0.020	0.016	0.013	0.020	0.016	0.015	0.013	0.012	0.0094
Range	0.0091	0.014	0.011	0.014	0.014	0.010	0.015	0.012	0.011	0.0097	0.0097	0.0078
Mean	0.011	0.011	0.010	0.010	0.0083	0.0078	0.0095	0.0085	0.0076	0.0072	0.0054	0.0053
SD	0.0026	0.0031	0.0027	0.0028	0.0033	0.0025	0.0037	0.0033	0.0028	0.0027	0.0022	0.0021
%CV	23.4	27.2	27.2	27.8	39.6	32.2	39.2	38.4	37.2	37.2	40.5	39.2
Outliers	0	1	0	1	0	0	1	1	0	2	0	1
Normality*	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Mann Whitney test	BIS70 vs RIS70		BIS80 vs RIS80		BIS90 vs RIS90		BIS60 vs RIS60		BIS70 vs RIS70		BIS80 vs RIS80	
P value (Exact)	0.9905		0.9846		0.5338		0,2503		0,5441		0,8953	
	22˚C						27˚C					
	RIS50	BIS50	RIS60	BIS60	RIS70	BIS70	RIS40	BIS40	RIS50	BIS50	RIS60	BIS60
Minimum	0.010	0.0096	0.0088	0.0088	0.0063	0.0062	0.014	0.013	0.013	0.012	0.013	0.012
Maximum	0.022	0.023	0.017	0.017	0.031	0.059	0.031	0.036	0.027	0.038	0.080	0.065
Range	0.012	0.013	0.0086	0.0084	0.025	0.053	0.017	0.024	0.014	0.026	0.067	0.052
Mean	0.015	0.015	0.013	0.013	0.013	0.020	0.021	0.021	0.020	0.020	0.031	0.029
SD	0.0028	0.0027	0.0022	0.0020	0.0056	0.015	0.0048	0.0060	0.0042	0.0060	0.019	0.017
%CV	18.2	18.0	17.3	15.8	43.9	76.1	22.9	29.1	21.1	30.6	60.3	56.5
Outliers	0	0	0	1	6	0	0	1	0	0	0	0
Normality*	Yes	Yes	Yes	Yes	No	No	Yes	Yes	No	No	No	Yes
Mann Whitney test	BIS50 vs RIS50		BIS60 vs RIS60		BIS70 vs RIS70		BIS40 vs RIS40		BIS50 vs RIS50		BIS60 vs RIS60	
P value	0.8920		0.6542		0.7791		0.4812		0.5594		0.6837	

	32˚C						37˚C					
	RIS30	BIS30	RIS40	BIS40	RIS50	BIS50	RIS20	BIS20	RIS30	BIS30	RIS40	BIS40
Minimum	0.021	0.014	0.020	0.017	0.018	0.016	0.03471	0.03222	0.03339	0.02968	0.03179	0.03
Maximum	0.049	0.047	0.047	0.060	0.047	0.049	0.08794	0.07444	0.08556	0.07064	0.08302	0.06841
Range	0.028	0.033	0.028	0.043	0.029	0.033	0.05323	0.04222	0.05217	0.04095	0.05122	0.04095
Mean	0.032	0.030	0.031	0.030	0.029	0.029	0.05352	0.0448	0.04834	0.0438	0.04871	0.04065
SD	0.0077	0.0084	0.0073	0.0098	0.0073	0.0092	0.01454	0.01251	0.01309	0.01067	0.01408	0.01177
%CV	24.1	28.1	24.0	32.8	24.7	32.2	27.17%	27.91%	27.08%	24.37%	28.91%	28.96%
Outliers	0	0	0	1	0	1	1	7	1	4	1	7
Normality	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No	Yes	No	No
Mann Whitney test	BIS30 vs RIS30		BIS40 vs RIS40		BIS50 vs RIS50		BIS20 vs RIS20		BIS30 vs RIS30		BIS40 vs RIS40	
P value	0.2433		0.3799		0.4813		0.0139		0.1469		0.0134	

*D'Agostino & Pearson test

3.3 Effect of eggshell thickness on water loss

As described in Section 2.4, water loss was measured at different humidities and temperatures, taking eggshell thickness into account. The analyses of results are presented in the following graphs, with supporting information in Table 3.3.

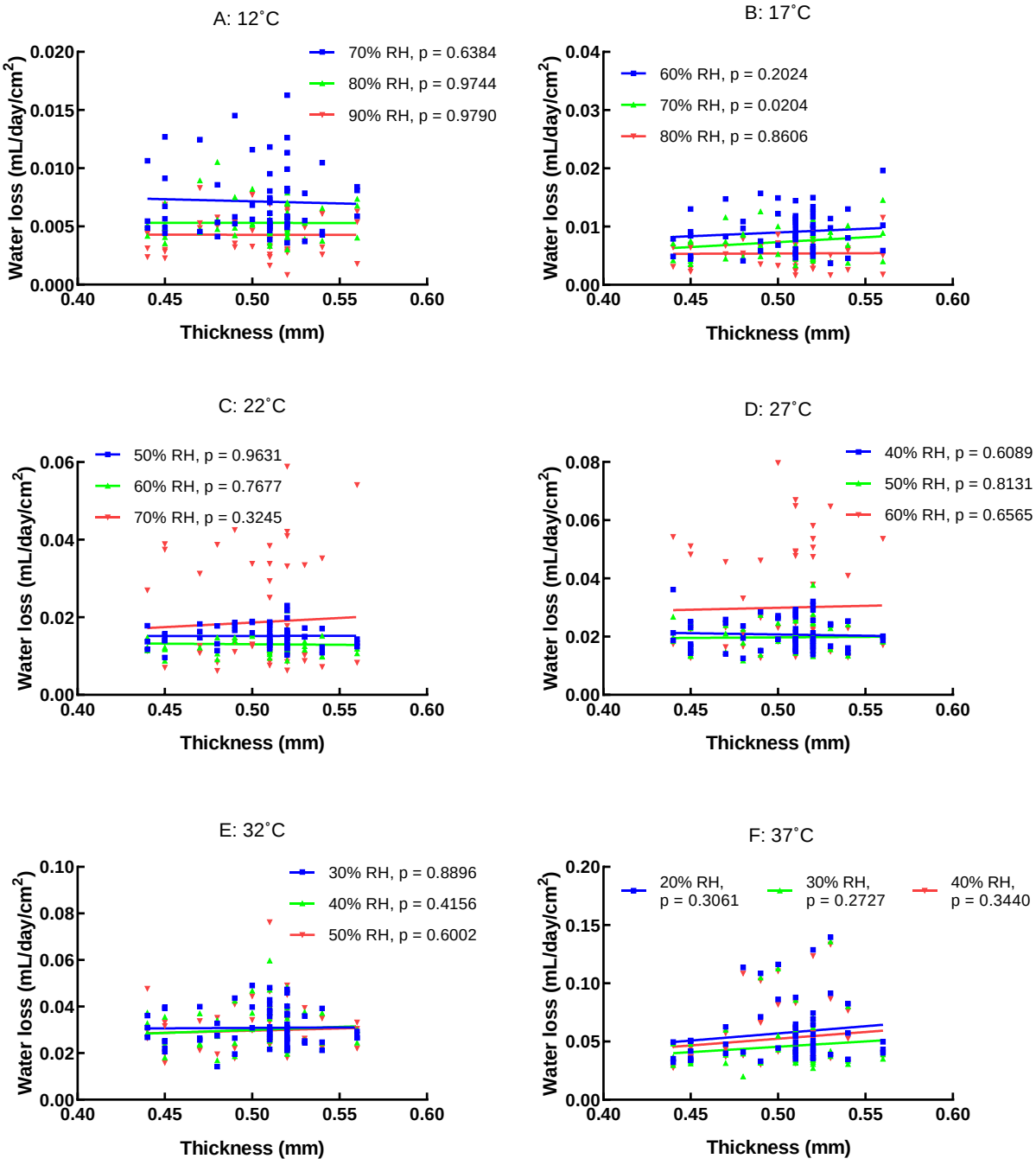


Figure 3.3.1: Linear regressions of water loss across the sixty eggshell fragments at different thicknesses. The water loss occurs over one day per square centimetre for different eggshell thicknesses as well as different temperatures and relative humidities. A: Water loss at 12˚C with 70%, 80% and 90% relative humidities. B: Water loss at 17˚C with 60%, 70% and 80% relative humidities. C: Water loss at 22˚C with 50%, 60% and 70% relative humidities. D: water loss at 27˚C with 40%, 50% and 60% relative humidities. E: Water loss at 32˚C with 30%, 40% and 50% relative humidities. F: Water loss at 37˚C with 20%, 30% and 40% relative humidities. The p-values for the regression lines are indicated and normal distribution are indicated in Table 3.3.

Figure 3.3.1 A: At 12˚C, there were no significant regressions at the three relative humidities. The D'Agostino & Pearson test for normality concluded that the data were normally distributed for all three humidities. The regression slopes were not significantly parallel with each other (p = 0.9067). The horizontal separation between the regression lines were, however, highly significantly different (<0.0001).

Figure 3.3.1 B: For 17˚C there were no significant regressions at the three relative humidities. The D'Agostino & Pearson test for normality concluded that the data were normally distributed for all three humidities. The regression slopes were not significantly parallel with each other (p = 0.3130). The horizontal separation between the regression lines were, however, highly significantly different (<0.0001).

Figure 3.3.1 C: For 22˚C there were no significant regressions at the three relative humidities. The D'Agostino & Pearson test for normality concluded that the data were normally distributed for all three humidities. The regression slopes were not significantly parallel with each other (p = 0.4469). The horizontal separation between the regression lines were, however, highly significantly different (<0.0001).

Figure 3.3.1 D: For 27˚C there were no significant regressions at the three relative humidities. The D'Agostino & Pearson test for normality concluded that the data were normally distributed for all three humidities. The regression slopes were not significantly parallel with each other (p = 0.7687). The horizontal separation between the regression lines were, however, highly significantly different (<0.0001).

Figure 3.3.1 E: For 32˚C there were no significant regressions at the three relative humidities. The D'Agostino & Pearson test for normality concluded that the data were normally distributed for all three humidities. The regression slopes were not significantly parallel with each other (p = 0.6803). The horizontal separation between the regression lines were, however, not significantly different (p = 0.8821).

Figure 3.3.1 F: For 37˚C there were no significant regressions at the three relative humidities. The D'Agostino & Pearson test for normality concluded that the data were normally distributed for all three humidities. The regression slopes were not significantly parallel with each other (p = 0.9770). The horizontal separation between the regression lines were, however, significantly different (p = 0.0431).

Table 3.3: Results and metrics of linear regressions for water loss at different temperature and relative humidity (%RH) treatments.

	12˚C			17˚C			22˚C		
	70%RH	80%RH	90%RH	60%RH	70%RH	80%RH	50%RH	60%RH	70%RH
Minimum	0.0056	0.0045	0.0031	0.0069	0.0054	0.0035	0.013	0.012	0.013
Maximum	0.0090	0.0069	0.0062	0.012	0.0092	0.0064	0.018	0.016	0.026
Range	0.0034	0.0024	0.0031	0.0051	0.0037	0.0028	0.0048	0.0041	0.013
Mean	0.0071	0.0053	0.0043	0.0091	0.0074	0.0054	0.015	0.013	0.019
SD	0.0010	0.00068	0.00081	0.0013	0.0010	0.00075	0.0015	0.0013	0.0032
%CV	14.09	12.76	19.02	14.9	13.8	13.9	9.8	9.7	16.9
Outliers	2	1	2	2	2	0	0	0	0
Normality*	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Slope	0.0076	0.0051	0.0062	0.0097	0.0066	0.0056	0.011	0.0096	0.024
Y-intercept	0.0038	0.0026	0.0031	0.0049	0.0033	0.0028	0.0057	0.0048	0.012
R squared	0.013	0.000059	0.000040	0.089	0.26	0.0018	0.00012	0.0050	0.054
P value	0.64	0.97	0.9790	0.2024	0.0204	0.8606	0.9631	0.7677	0.3245
Slopes equal (p)			0.9067			0.3130			0.4469
Elevations equal (p)		**<0.0001**			**<0.0001**			**<0.0001**	

	27˚C			32˚C			37˚C		
	40%RH	50%RH	60%RH	30%RH	40%RH	50%RH	20%RH	30%RH	40%RH
Minimum	0.018	0.017	0.024	0.025	0.022	0.024	0.038	0.030	0.034
Maximum	0.025	0.024	0.040	0.040	0.038	0.043	0.090	0.072	0.085
Range	0.0077	0.0063	0.017	0.015	0.017	0.018	0.052	0.042	0.052
Mean	0.021	0.020	0.030	0.031	0.030	0.030	0.053	0.046	0.049
SD	0.0022	0.0020	0.0039	0.0037	0.0042	0.0047	0.014	0.011	0.014
%CV	10.7	10.0	13.1	12.0	13.9	15.7	27.10	24.12	29.40
Outliers	1	0	0	0	1	0	3	1	3
Normality*	**Yes**	**Yes**	No	No	**Yes**	No	No	**Yes**	No
Slope	0.017	0.015	0.029	0.028	0.031	0.035	0.12	0.092	0.12
Y-intercept	0.0084	0.0077	0.015	0.014	0.016	0.018	-0.0047	-0,00048	-0,0053
R squared	0.015	0.0032	0.011	0.0011	0.037	0.16	0.058	0.066	0.050
P value	0.6089	0.8131	0.6565	0.8896	0.4156	0.6002	0.3061	0.2727	0.3440
Slopes equal (p)		0.7687		0.8821			0.9770		
Elevations equal (p)		**<0.0001**			0.6803			**0.0431**	

*D'Agostino & Pearson test

3.4 Water loss at different humidities

As described in Section 2.4, water loss was measured at different humidities. The analyses of the ANOVA and Tuckey's post-tests are presented in Figure 3.4.1 A-F.

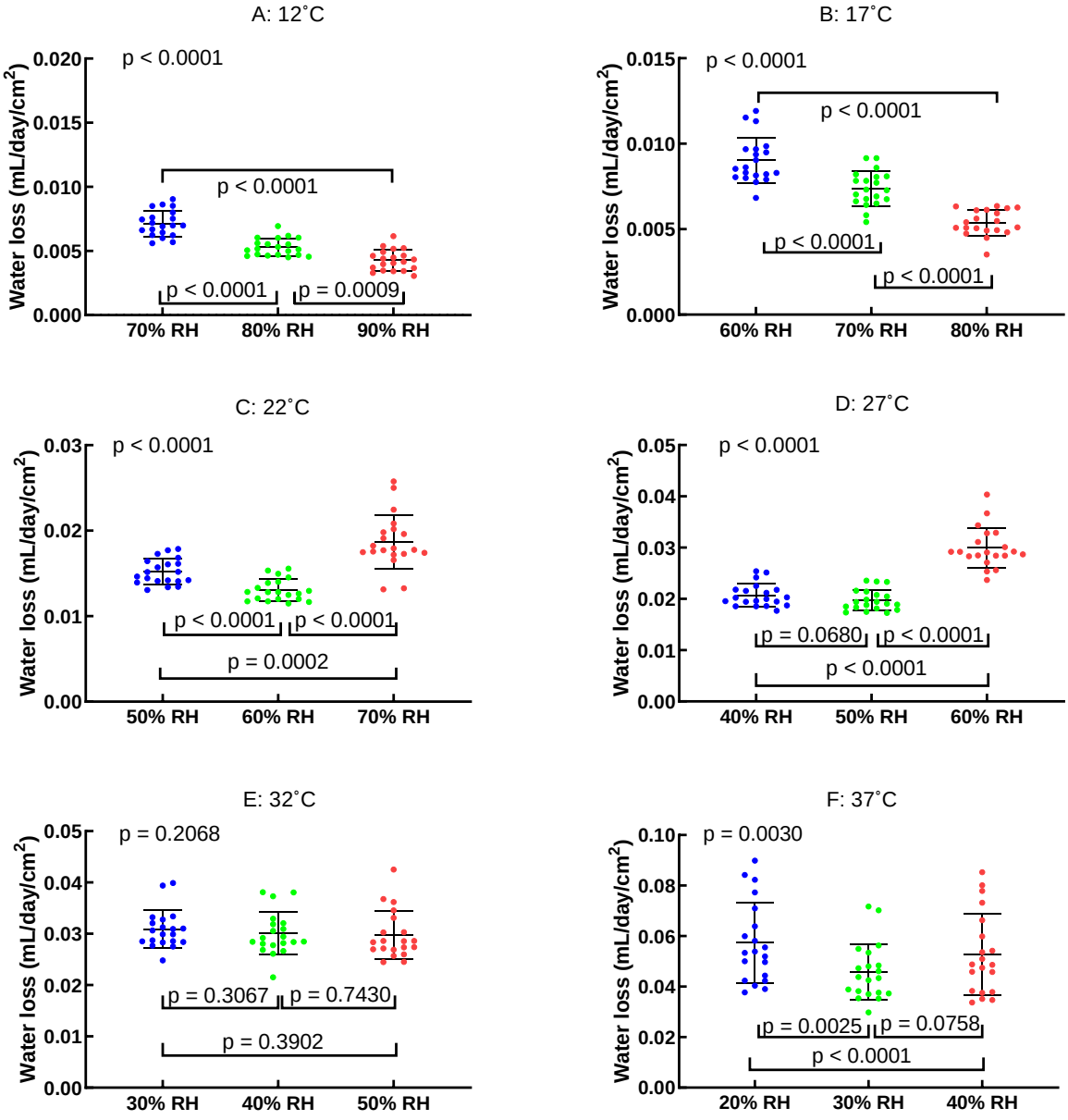


Figure 3.4.1: Scatter plot for water loss over 24 hours at different temperatures and relative humidities. The result of the ANOVA is presented at the top of each graph, and the results of the Tuckey's multiple comparison post-test is provided with paired brackets, in each graph. A: Water loss across eggshell fragments at 12˚C for 70%, 80% and 90% relative humidity. B: Water loss across eggshell fragments at 17˚C for 60%, 70% and 80% relative humidity. C: Water loss across eggshell fragments at 22˚C for 50%, 60% and 70% relative humidity. D: Water loss across eggshell fragments at 27˚C for 40%, 50% and 60% relative humidity. E: Water loss across eggshell fragments at 32˚C for 30%, 40% and 50% relative humidity. F: Water loss across eggshell fragments at 37˚C for 20%, 30% and 40% relative humidity. Significance are indicated on the graph with comparisons between the relative humidities (Tukey's comparisons test) as well as ANOVA summary.

Figure 3.4.1 A: Scatterplot comparing water loss at 12°C for 70%, 80%, and 90% relative humidity. All three humidities were normally distributed. There was a significant difference in water loss between the three relative humidities (ANOVA; $p < 0.0001$). The Tuckey's post-tests showed that all treatments differed significantly from each other.

Figure 3.4.1 B: Scatterplot comparing water loss at 17°C for 60%, 70% and 80% relative humidity. All three humidities were normally distributed. There was a significant difference in water loss between the three relative humidities (ANOVA; $p < 0.0001$). The Tuckey's post-tests showed that all treatments differed significantly from each other.

Figure 3.4.1 **C:** Scatterplot comparing water loss at 22°C for 50%, 60% and 70% relative humidity. All three humidities were normally distributed. There was a significant difference in water loss between the three relative humidities (ANOVA; $p < 0.0001$). The Tuckey's post-tests showed that all treatments differed significantly from each other.

Figure 3.4.1 D: Scatterplot comparing water loss at 27°C for 40%, 50% and 60% relative humidity. Both 40% and 50% relative humidities were normally distributed, however 60% was not normally distributed. The Tuckey's post-tests showed the comparison between 40% and 50% relative humidity where not significant, however the comparison between 40% and 60%, and 50% and 60% relative humidity were significant. All three humidities were normally distributed. There was a significant difference in water loss between the three relative humidities (ANOVA; $p < 0.0001$).

Figure 3.4.1 E: Scatterplot comparing water loss at 32°C for 30%, 40% and 60% relative humidity. Data that were not normally distributed for 30% and 50% relative humidity, but it was normally distributed for 40%. The Tuckey's post-tests showed the water loss for all three humidities were not significant. There was not a significant difference in water loss between the three relative humidities (ANOVA; ($p = 0.2068$).

Figure 3.4.1 F: Scatterplot comparing water loss at 37°C for 20%, 30% and 40% relative humidity. Data were normally distributed for all three humidities. The Tuckey's post-tests showed there is a significant difference in water loss between 20% and 30% as well as between 20% and 40% relative humidities, except there was no significant difference for the 30% and 40% comparison. There was a significant difference in water loss between the three relative humidities (ANOVA; $p = 0.0030$).

Water loss during the different temperature-humidity treatments was variable for every single treatment (Figure 3.4.2). Therefore, I looked at how the coefficients of variance (%CV) for water losses at different temperatures (Figure 3.4.2). These values are provided in Table 3.3 and plotted in Figure 3.4.2. The best non-linear regression model that described the line was the 'sum of two Lorentzian' distribution lines, with an R^2 of 0.86, which is a good fit.

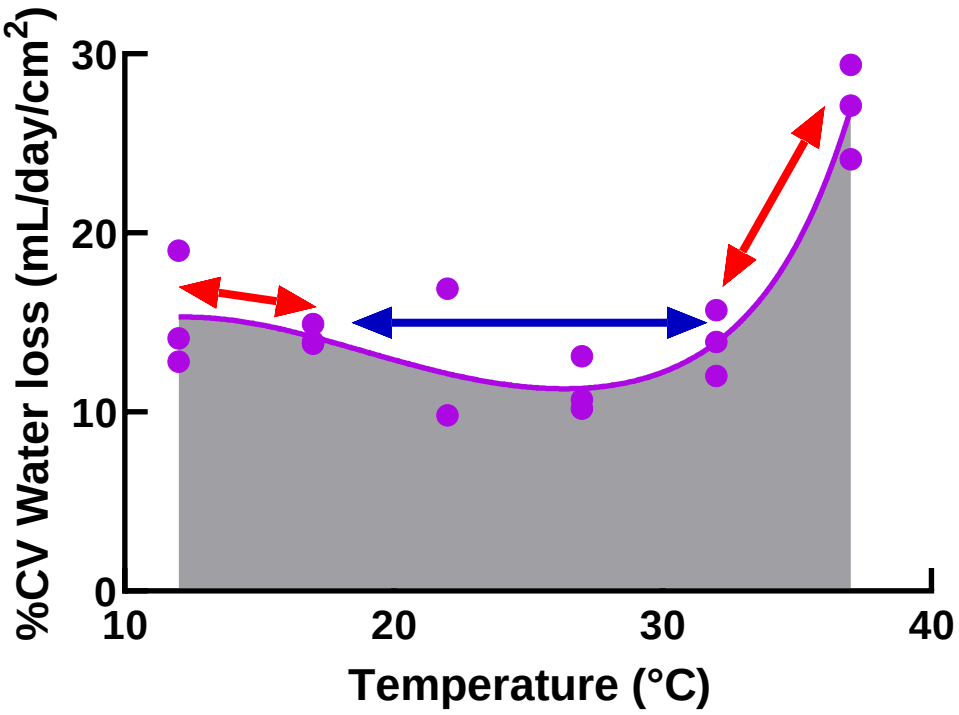


Figure 3.4.2: Scatterplot indicating coefficient variance for water loss at different temperatures. The fit of the points to the line of the 'sum of two Lorentzian model' was $R^2 = 0.86$. The blue arrow indicates the 15°C temperature window with lowest %CV.

3.5.1 Are pore densities and eggshell thickness related?

As discussed in Section 1.6.2, there is also the effect of pore densities on water loss to consider. Here, I present the results of the investigation of whether pore densities did indeed affect water loss, in relation to eggshell thickness. As was shown in Figure 3.2.2, there was no significant difference in pore densities in eggshells between the two islands, therefore allowing the pooling of the twenty eggs from both study sites into one dataset. Figure 3.5.2 shows the linear regression analyses with the assumption that eggshell thickness would be related to pore densities. Although there was a trend of decreasing pore density with thicker shells, this trend was not significant for this dataset. Therefore, pore density need to be considered as an independent factor when measuring the effect of temperature and humidity on water loss.

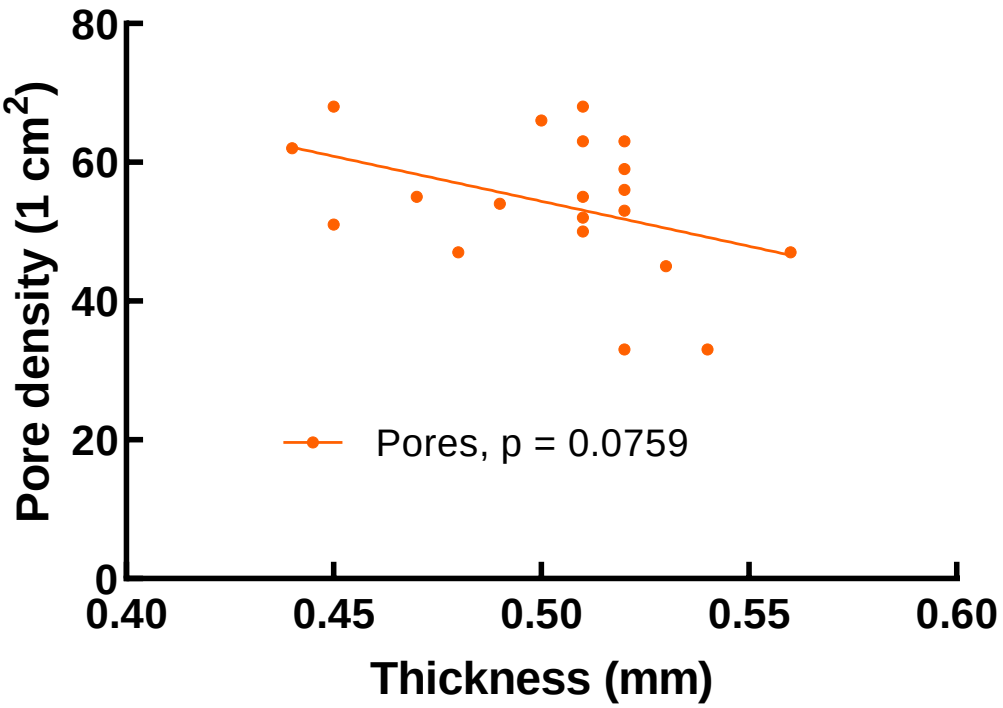


Figure 3.5.1: Regression of normally distributed pore density against thickness. Pore density decreased with an increase in eggshell thickness, but this was not significant.

3.5.2 Effect of pore densities on water loss

Water loss may be affected by pore densities independent of eggshell thickness in an African Penguin eggshell. I therefore investigated whether water loss increased or decreased with an increase in pore density at different temperatures and humidities, using the same data as in Section 3.3.

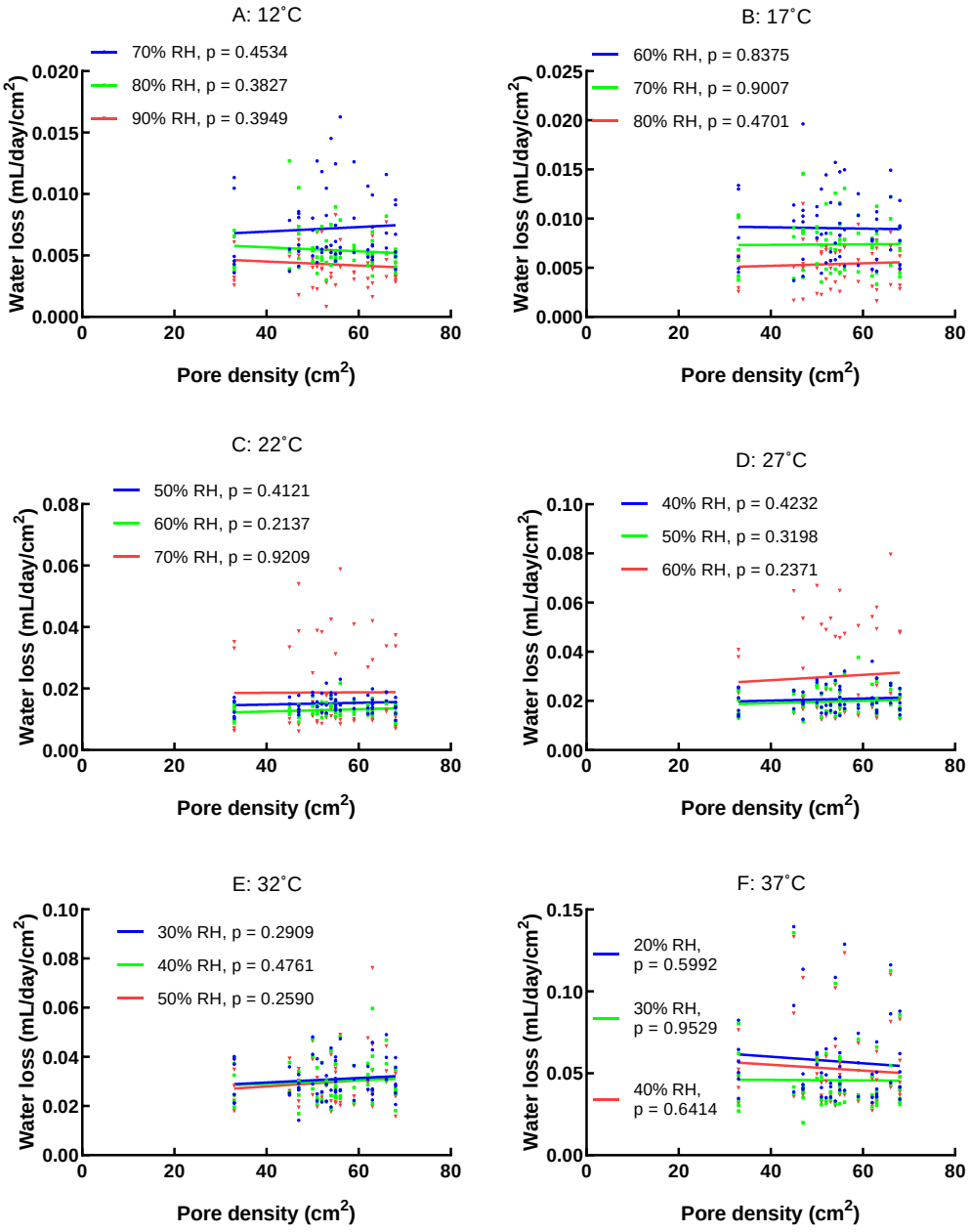


Figure 3.5.2: Linear regressions for water loss over 24 hours per square centimetre for different pore densities for all 60 eggshell fragments. A: Water loss across eggshell fragments at 12˚C for 70%, 80%, and 90% relative humidity. B: Water loss across eggshell fragments at 17˚C for 60%, 70%, and 80% relative humidity. C: Water loss across eggshell fragments at 22˚C for 50%, 60%, and 70% relative humidity. D: Water loss across eggshell fragments at 27˚C for 40%, 50%, and 60% relative humidity. E: Water loss across eggshell fragments at 32˚C for 30%, 40%, and 50% relative humidity. F: Water loss across eggshell fragments at 37˚C for 20%, 30%, and 40% relative humidity. The p-values for each regression line are indicated.

Figure 3.5.2 A: For 12˚C, there was no significant effect of pore density on water loss for the three humidities (70%, 80%, and 90%). The D'Agostino & Pearson normality test concluded that for 12˚C all the data were normally distributed for all three humidities. The horizontal separation between the regression lines are significantly different ($p < 0.0001$), but the slopes were not significant ($p = 0.3909$).

Figure 3.5.2 B: For 17˚C, there was no significant effect of pore density on water loss for the three humidities (60%, 70%, and 80%). The D'Agostino & Pearson normality test concluded that for 17˚C all the data were normally distributed for all three humidities. The horizontal separation between the regression lines are significantly different ($p < 0.0001$), but the slopes were not significant ($p = 0.8610$).

Figure 3.5.2 C: For 22˚C, there was no significant effect of pore density on water loss for the three humidities (50%, 60%, and 70%). The D'Agostino & Pearson normality test concluded that for 22˚C all the data were normally distributed for all three humidities. The horizontal separation between the regression lines are significantly different ($p < 0.0001$), but the slopes were not significant ($p = 0.9124$).

Figure 3.5.2 D: For 27˚C, there was no significant effect of pore density on water loss for the three humidities (40%, 50%, and 60%). The D'Agostino & Pearson normality test concluded that for 27˚C the data were normally distributed for 40% and 50% relative humidity, except for 60% relative humidity. The horizontal separation between the regression lines are significantly different ($p < 0.0001$), but the slopes were not significant ($p = 0.7242$).

Figure 3.5.2 E: For 32˚C, there was no significant effect of pore density on water loss for the three humidities (30%, 40%, and 50%). The D'Agostino & Pearson normality test concluded that for 32˚C the data were normally distributed for 40% relative humidity, except for 30% and 50% relative humidity. The horizontal separation between the regression lines are not significantly different ($p = 0.6702$), and the slopes were not significant ($p = 0.9255$).

Figure 3.5.2 F: For 37˚C, there was no significant effect of pore density on water loss for the three humidities (70%, 80%, and 90%). The D'Agostino & Pearson normality concluded that for 37˚C all the data were normally distributed for all three humidities. The horizontal separation between the regression lines were significantly different ($p = 0.0497$), but the slopes were not significant ($p = 0.9169$).

Table 3.4: Results and metrics of linear regressions for water loss at different temperature and relative humidity (RH%) treatments with different pore densities.

	12˚C			17˚C			22˚C		
	70%RH	80%RH	90%RH	60%RH	70%RH	80%RH	50%RH	60%RH	70%RH
Minimum	0.0056	0.0045	0.0031	0.0068	0.0054	0.0035	0.013	0.012	0.013
Maximum	0.0090	0.0073	0.0062	0.012	0.0092	0.0064	0.018	0.016	0.026
Range	0.0034	0.0028	0.0031	0.0051	0.0037	0.0028	0.0048	0.0041	0.0123
Mean	0.0072	0.0054	0.0043	0.0090	0.0074	0.0054	0.015	0.013	0.019
SD	0.0010	0.00079	0.00081	0.0013	0.0010	0.00075	0.0014	0.0013	0.0032
%CV	14.3	14.5	19	14.9	13.8	13.9	9.8	9.7	16.9
Outliers	1	0	2	2	2	0	0	0	0
Normality*	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Slope	0.000023	0.000016	0.000019	0.000032	0.000024	0.000017	0.000034	0.000029	0.000075
Y-intercept	0.0013	0.00088	0.0010	0.0017	0.0013	0.00095	0.0019	0.0016	0.0041
R squared	0.035	0.0026	0.041	0.0024	0.00089	0.029	0.038	0.085	0.00057
P value	0.4273	0.8309	0.3949	0.8375	0.9007	0.4701	0.4121	0.2137	0.9209
Slopes equal (p)		0.4405			0.861			0.9124	
Elevations equal (p)		**<0.0001**			**<0.0001**			**<0.0001**	

	27˚C			32˚C			37˚C	
	40%RH	50%RH	60%RH	30%RH	40%RH	50%RH	20%RH	30%RH	40%RH
Minimum	0.018	0.017	0.024	0.025	0.022	0.024	0.038	0.030	0.034
Maximum	0.025	0.024	0.040	0.040	0.038	0.043	0.090	0.072	0.085
Range	0.0077	0.0063	0.0167	0.015	0.017	0.018	0.052	0.042	0.052
Mean	0.021	0.020	0.030	0.031	0.030	0.030	0.057	0.046	0.053
SD	0.0022	0.0020	0.0039	0.0037	0.0042	0.0047	0.016	0.011	0.016
%CV	10.7	10.2	13.1	12	13.9	15.8	27.81	24.27	30.51
Outliers	1	0	0	0	1	0	2	2	2
Normality*	**Yes**	**Yes**	No	No	**Yes**	No	**Yes**	**Yes**	**Yes**
Slope	0.000051	0.000046	0.000089	0.000085	0.000098	0.00010	0.00037	0.00026	0.00038
Y-intercept	0.0028	0.0025	0.0049	0.0046	0.0053	0.0058	0.021	0.014	0.020
R squared	0.036	0.055	0.077	0.062	0.029	0.070	0.016	0.00020	0.012
P value	0.4232	0.3198	0.2371	0.2909	0.4761	0.259	0.5992	0.9529	0.6412
Slopes equal (p)		0.7242			0.9255			0.9169	
Elevations equal (p)		**<0.0001**			0.6702			**0.0497**	

3.6 Water loss for the same humidity at different temperatures

As described in Section 2.4, water loss was measured at different humidities and temperatures. Here, I use the same data as in Section 3.3 for temperature, but now according to humidity, investigating the effect of eggshell thickness on water loss. The results are presented in Figure 3.6 A-D, with supporting information in Table 3.5.

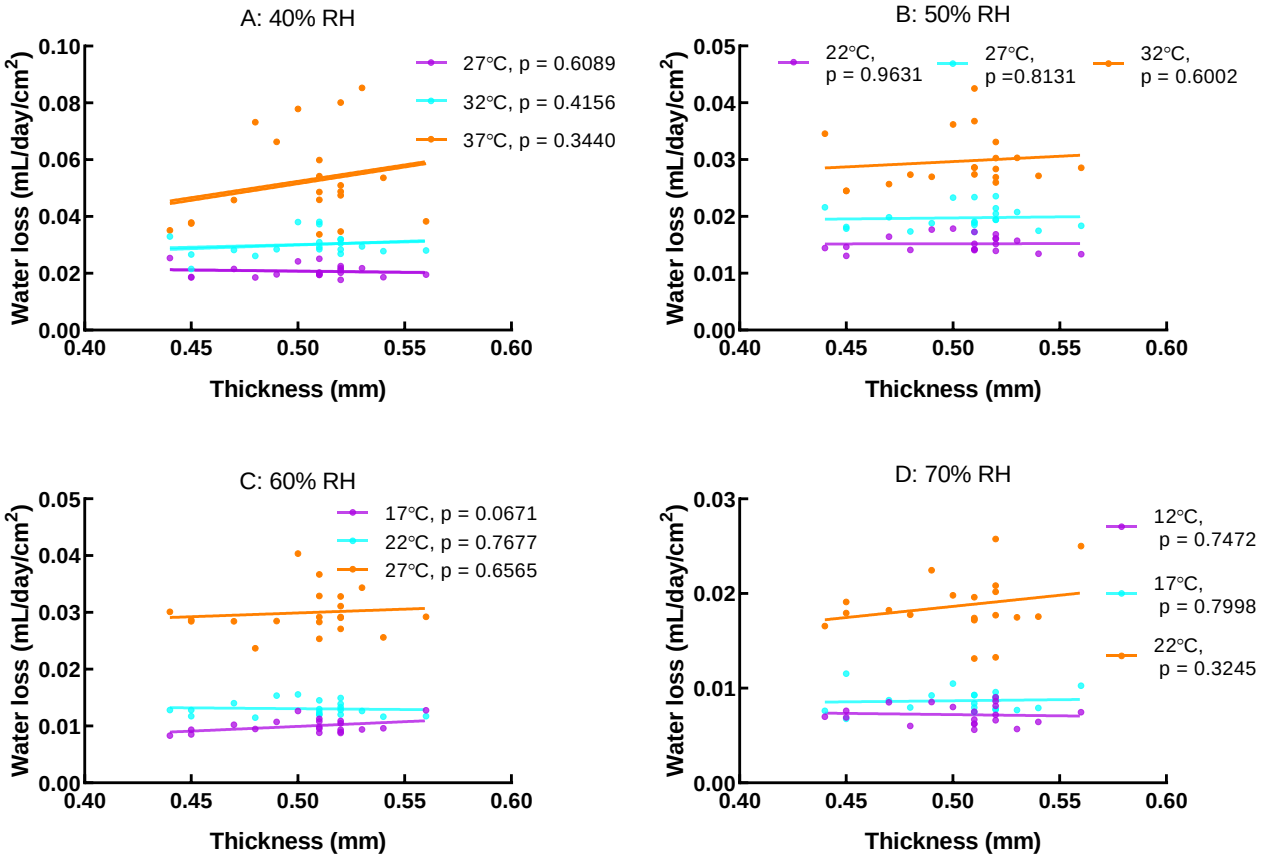


Figure 3.6.1: Linear regressions for water loss over 24 hours at different temperatures and relative humidities, according to eggshell thickness. A: Water loss across eggshell fragments at 40% relative humidity for 27˚C, 32˚C, and 37˚C. B: Water loss across eggshell fragments at 50% relative humidity for 22˚C, 27˚C, and 32˚C. C: Water loss across eggshell fragments at 60% relative humidity for 17˚C, 22˚C, and 27˚C. D: Water loss across eggshell fragments at 70% relative humidity for 12˚C, 17˚C, and 22˚C. Standard deviations are indicated in Table 3.5. The p-values for the regressions are indicated.

Figure 3.6.1 A: At 40% relative humidity, there was no significant effect of eggshell thickness on water loss at the three temperatures (27˚C, 32˚C, and 37˚C). The horizontal separation between the regression lines was highly significant ($p < 0.0001$). The slopes of the regression lines were however, not significantly different ($p = 0.4535$).

Figure 3.6.1 B: At 50% relative humidity, there was no significant effect of eggshell thickness on water loss at the three temperatures (22˚C, 27˚C, and 32˚C). The horizontal

separation between the regression lines was highly significant ($p < 0.0001$). The slopes of the regression lines were however, not significantly different ($p = 0.8370$).

Figure 3.6.1 C: At 60% relative humidity, there was no significant effect of eggshell thickness on water loss at the three temperatures (17˚C, 22˚C, and 27˚C). The horizontal separation between the regression lines was highly significant ($p < 0.0001$). The slopes of the regression lines were however, not significantly different ($p = 0.7308$).

Figure 3.6.1 D: At 70% relative humidity, there was no significant effect of eggshell thickness on water loss at the three temperatures (12˚C, 17˚C, and 22˚C). The horizontal separation between the regression lines was highly significant ($p < 0.0001$). The slopes of the regression lines were however, not significantly different ($p = 0.4334$).

Table 3.5: Results and metrics of linear regressions for different relative humidities (RH%) treatments comparing water loss at different temperatures.

	40%			50%			60%			70%		
	27˚C	32˚C	37˚C	22˚C	27˚C	32˚C	17˚C	22˚C	27˚C	12˚C	17˚C	22˚C
Minimum	0.0177	0.02151	0.03368	0.01306	0.01727	0.024	0.0083	0.012	0.024	0.0056	0.0068	0.013
Maximum	0.02538	0.0381	0.08533	0.01785	0.02356	0.043	0.013	0.016	0.040	0.0090	0.012	0.026
Range	0.007681	0.01659	0.05165	0.00479	0.006296	0.018	0.0044	0.0041	0.017	0.0034	0.0048	0.013
Mean	0.02072	0.03005	0.04872	0.01519	0.01975	0.030	0.010	0.013	0.030	0.0072	0.0087	0.019
SD	0.002212	0.00417	0.01432	0.001482	0.002019	0.0047	0.0012	0.0013	0.0039	0.0010	0.0012	0.0032
%CV	10.68	13.88	29.40	9.757	10.22	15.7	12.4	9.7	13.1	14.3	13.6	16.9
Outliers	1	1	5	0	0	0	1	0	0	1	2	0
Normality*	**Yes**	No	No	**Yes**	**Yes**	No	**Yes**	**Yes**	No	**Yes**	**Yes**	**Yes**
Slope	0.01662	0.03098	0.1188	0.01122	0.01526	0.035	0.0085	0.010	0.029	0.0078	0.0089	0.023
Y-intercept	0.008377	0.01561	0.05985	0.005654	0.007689	0.018	0.0043	0.0048	0.015	0.0039	0.0045	0.012
R squared	0.01484	0.03715	0.04987	0.000122	0.003188	0.016	0.17	0.0050	0.011	0.0059	0.0037	0.054
P value	0.6089	0.4156	0.344	0.9631	0.8131	0.60	0.067	0.77	0.66	0.75	0.80	0.32
Slopes equal (p)		0.4535			0.837			0.7308			0.4334	
Elevations equal (p)		**<0.0001**			**<0.0001**			**<0.0001**			**<0.0001**	

3.7 Water loss between clean (washed) and dirty (EEM-covered) eggs

Since the eggs were found covered in EEM, there was a difference in eggshell thickness before and after they were washed. Therefore, I investigated if the presence and absence of EEM would influence water loss, using two sets of seven 'clean' and 'dirty' eggshell fragments. Due to lack of enough eggs, one set of seven fragments were from a 'clean' and washed egg, and the seven 'dirty' fragments were from another 'dirty' unwashed egg. The thickness of each fragment was measured, as in Section 2.2. As can be expected, the 'clean' eggs were significantly thinner (0.61 mm) than the 'dirty' eggs (0.63 mm) ($p = 0.0252$; Mann-Whitney, unpaired, two-tailed, test).

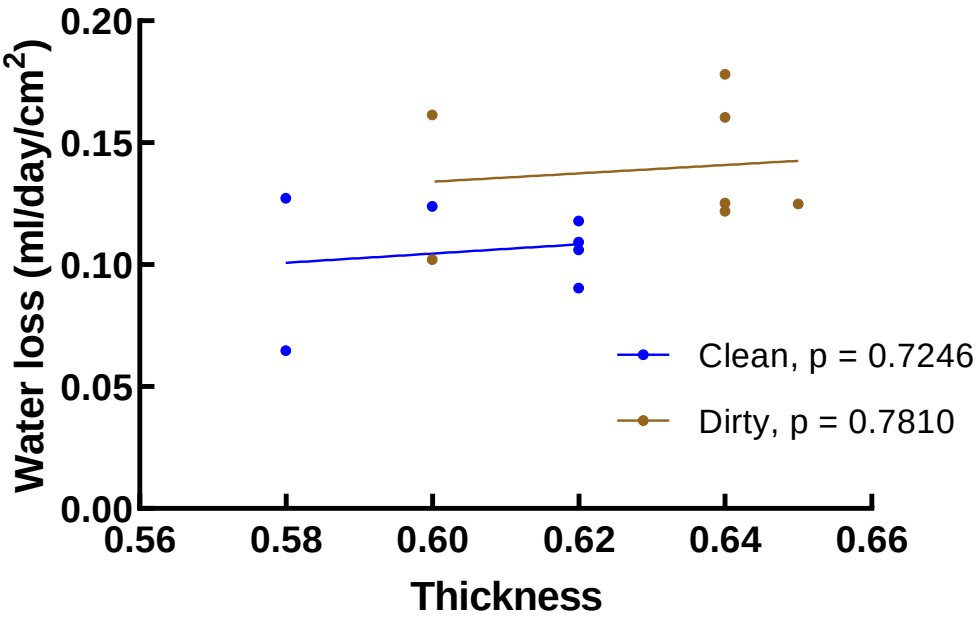


Figure 3.7.1: Linear regression of normally distributed water loss with thickness for clean and dirty (EEM- covered) eggshells.

Figure 3.7.1 shows that the slopes of water loss for both sets of shells were not significantly influenced by their own thickness, nor were the slopes significantly different from each other ($p=0.9831$). The horizontal separation between the two slopes were also not significantly different ($p = 0.1035$). Therefore, the difference in eggshell thickness, although significant ($p = 0.0252$; Mann-Whitney, unpaired, two-tailed, test), did not have an effect on water loss. What would be the effect on water loss if only 'clean' and 'dirty' shells were compared, the comparison of which is valid since the effect of thickness was not significant.

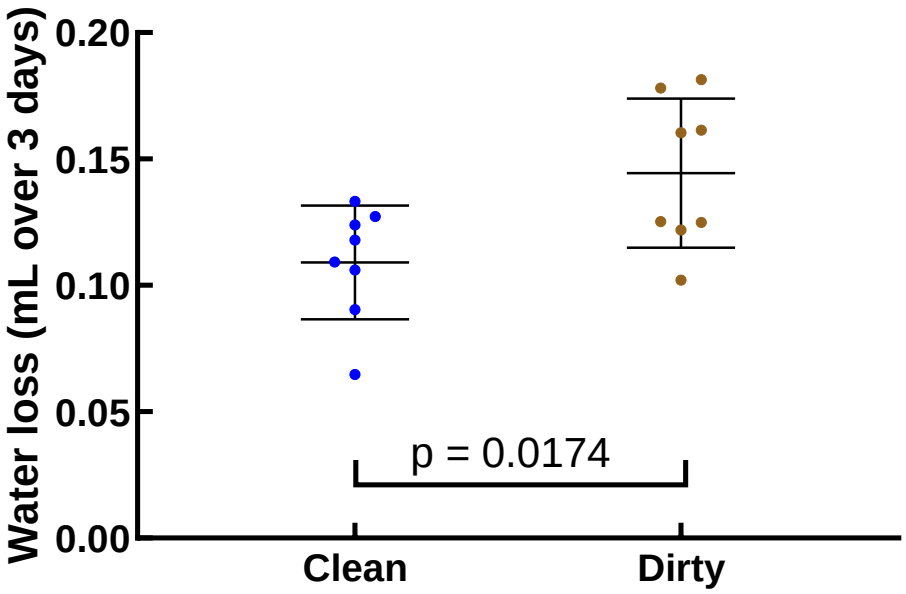


Figure 3.7.2: Scatter plot of water loss for clean (washed) and dirty (EEM-covered) eggshells. There was a significant difference.

Figure 3.7.2 shows that 'dirty' eggs, despite being significantly thicker, also lost significantly more water than the 'cleaner' thinner shells.

3.8 Comparison of water loss between chicken and African Penguin eggshells

As discussed in Section 2.7, water loss through chicken eggshells was investigated in a previous study done by Veldsman et al. (2020). The water loss pattern through chicken eggshells is compared with the water loss pattern for the African Penguin eggshell (Figure 3.8.1). Although the Y-axes differ in expression between the two columns of graphs, the two columns are comparable, and only the slopes of the regression lines need to be compared. Comparable temperature and relative humidity treatments from both studies were placed next to each other.

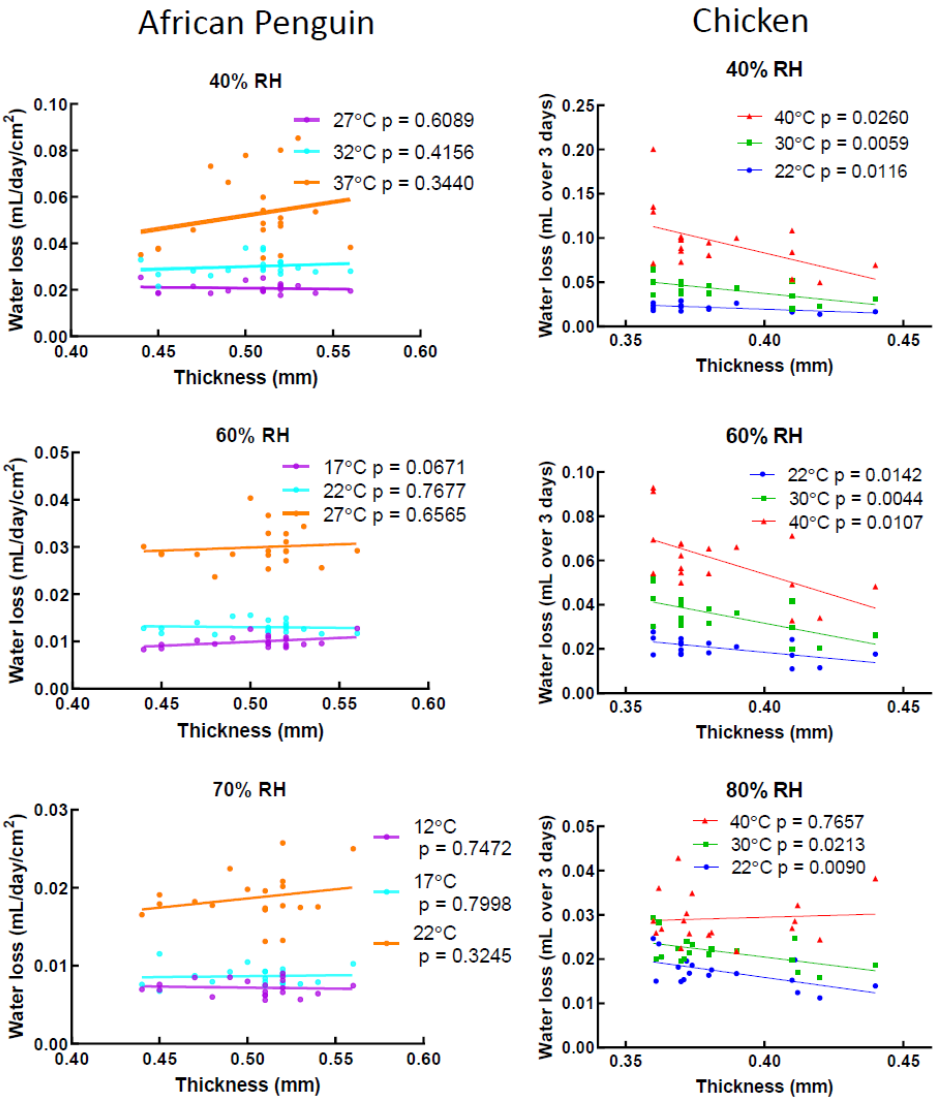
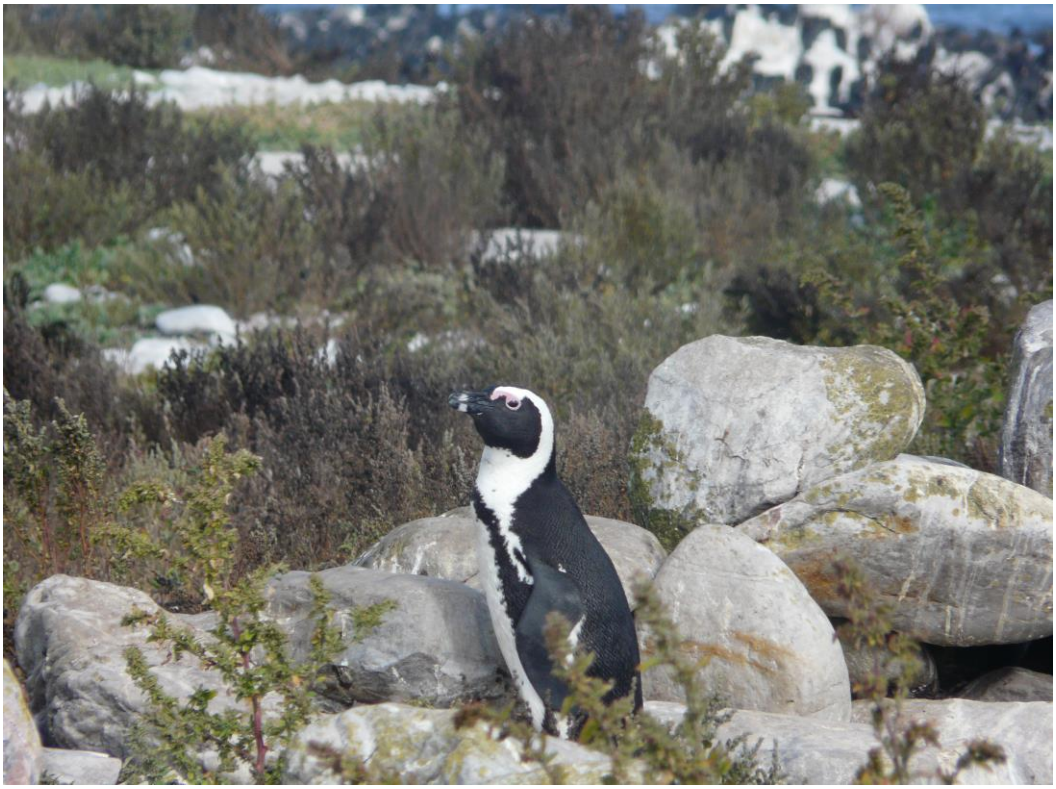


Figure 3.8.1: Comparison between water loss patterns for chicken eggshells and African Penguin eggshells, in left and right columns.

Comparing the left hand column (penguin) water loss patterns with the corresponding right hand column (chicken), shows that water loss through chicken eggshells in almost all treatments decreased significantly with an increase in eggshell thickness at all except one treatment. This was not the case for water loss through penguin eggshells.



“The greatness of a nation can be judged by the way its animals are treated”
-Mahatma Gandhi

CHAPTER 4: DISCUSSION

4.1 Eggshell characteristics

This study is a relatively new approach especially the investigation of water loss through different eggshell thicknesses of the African Penguin relative to eggshell thickness. Other studies investigated whole egg mass loss between different species without considering eggshell thickness (Nakage et al. 2003, Deeming 2011). Bouwman et al. (2015) used the egg contents of twenty African Penguin eggs for POPs analyses. The remaining eggshell fragments were stored and thus available for this study. This use therefore ethically extended the usefulness of materials from an Endangered species.

The thickness of the African penguin eggshell has received little study in two crucial aspects. Firstly, how external climatic factors influence water vapour pressure and secondly, how pollution affects water loss by influencing thickness and pore density. Such influences may result in reduced structural performance of the eggshell and affect embryo development due to enhanced water loss, leading to an even more reduced penguin population. Previous studies found that eggshell characteristics are the only part that evolves as a unique adaption to regulate water loss from bird eggs (El-Tarabany 2016). Due to pollution however, the process that forms the bird eggshell has been compromised and the eggshells of some bird species are even thinner now than before.

In Table 3.1, a summary of the African Penguin eggshell characteristics was given. This data gave an overall view of characteristics of the shells used for this study. When these results were compared to previous studies, the pore density for this study was 32% higher than a previous study (Amos & Rahn 1985). Mean eggshell thickness for this study was thinner in comparison with other studies (Ar & Rahn. 1985; Klusener et al. 2018). I do not know the reason for this, but it may have to do with variable calcium availability to the females during different years. However, the rest of the eggshell characteristics seemed to be almost the same as the previous studies.

4.2 Difference between eggs from Robben Island and Bird Island

Even though there is a distance of more than 650 km between the two breeding colonies from Robben Island and Bird Island, there were no significant morphometric differences between the two groups of eggshells (Section 3.2).

Firstly, there was no significant difference between the two different islands in eggshell thickness (Figure 3.2.1). Secondly, there was no significant difference in pore density of eggshells from the two islands (Figure 3.2.2). Both these findings made it possible to compare the water loss through eggshell thickness as well as water loss through pore density between Robben Island and Bird Island eggshells.

The rate of mass loss for each test unit during the different weighing periods reflects the rate of water loss during the different temperature-humidity treatments. Figure 3.2.3 showed no significant difference in water loss between the compared relative humidities at specific temperatures between the islands, except for 37˚C at 20% and 40% relative humidity were a significant difference was found.

The 37°C treatment was the highest temperature treatment representing predicted future conditions that may be experienced due to climate change. One reason for this could be due to, as expected for future climate changes, higher than ambient temperature and lower humidities resulting in unpredictable and very high water losses for African Penguin eggs. Why at this temperature there was a significant difference between the two islands is not clear, but may indicate that stress conditions (climate change) beyond normal may affect the colonies differently. The significant difference in water loss was also seen at 20% and 40% relative humidity, but not at 30%. Strange is also that some eggs from Bird Island seem to lose much more water than the others from the same island while the mean water loss was lower than the mean for eggs from Robben Island. This suggests that the effects of temperature stress affect some eggs more than other eggs, and then only at the higher (40%) and lower (20%) humidity, but not at the intermediate relative humidity of 30%. I will come back to this phenomenon in Section 4.4.

Despite the differences seen at 37°C, where there were significant differences at 20% and 40% relative humidity, I concluded that this does not present enough reason to keep the data sets separate for the two islands, and continued with results for all twenty eggs pooled.

4.3 Effect of eggshell thickness on water loss

For the mass of the African Penguin egg, water loss is expected to approximate 0.37-0.56 ml per day during incubation (Klusener et al.2018). The mean water loss measured for this study (0.421 ml/day) was slightly higher than measured for African Penguin eggshell fragments used by another study. Yom-Tov, et al. (1986) found 0.411 ml/day at 35°C. The water loss differences between these two studies and mine could be because of two reasons – 1) the absence of the membrane in this study and, 2) I removed the eggshell extraneous matter (EEM) from the outer surface of the eggshell. Mittal et al. (2016) found that the membrane regulated water loss from fresh eggs (the membrane was already removed from the present eggshells by Bouwman et al. (2015)). EEM was removed for this study as eggs had vastly different EEM coverage that also differed across individual eggshell surface. Leaving the EEM on would have introduced variability independent of each egg. I did measure the effect of EEM on water loss, and will return to this factor in Section 4.7.

For all temperature treatments, there were no differences in the slopes or rate of loss affected by eggshell thickness (Figure 3.3.1). This means that irrespective of eggshell thickness, the rate of water loss (i.e. the slope) at any temperature and humidity treatment was not affected by eggshell thickness. However, the amount of loss was different at

different temperature treatments ($p < 0.05$ for horizontal separation between the regression lines) between 12˚C and 27˚C, but at the elevated (climate change) temperatures, the slopes were not distinguishable by separation. These differences will be further explored in Section 4.4.

I initially predicted that there would be a significant association between eggshell thickness and water loss, as previously tested and seen in chicken eggshells (Veldsman et al. 2020). In the Veldsman et al. (2020) study, I found that thicker chicken shells lost water at a significantly slower rate than thinner shells, at any temperature and humidity combination. Unexpectedly therefore, this was not observed for the African Penguin eggshells. I will discuss the possible reasons for the differences between the chicken and penguin egg responses in Section 4.8.

Since no other studies have looked at the combined effects of different temperature, humidity, and eggshell thicknesses, especially on African Penguin eggs, comparisons with existing knowledge is not possible.

4.4 Water loss at different humidities

Since I found that eggshell thickness did not affect rate of water loss for any given temperature and humidity treatment, this factor can be ignored when looking at water loss affected by humidity. As explained in Section 2.4, the same humidity settings at each temperature would not be representative of actual measurements at Cape Town. Therefore, the lowest humidity at each temperature decreased with an increase in temperature, reflecting actual and expected conditions. Direct comparisons between the temperature treatments are possible, but this distinction should be kept in mind.

The results, graphically presented in Figure 3.4.1 presents a complex dynamic. Based on the ANOVA results, water loss decreased significantly and apparently linearly with increased humidity at 12°C and 17°C. At 22°C and 27°C however, the intermediate humidity of each treatment had the lowest water loss rate compared with the highest and lowest humidity – an U-shaped effect. At the 32°C treatment (at a climate change temperature), the linear decrease became apparent again (although not significant), and the U-shape returned at 37°C, with a complex set of significant and non-significant differences between the humidity treatments.

At the lower temperatures (12°C and 17°C), it seems that the shells loose water faster at lower humidities, while at 22°C and 27°C more water is lost at higher humidities. Higher water lost at lower temperatures and humidities suggest that the ambient humidity regulates water loss – the less water in the air outside, the more water is lost. However, at higher temperatures, more water is lost at higher humidities. This may suggest a cooling effect, since, just like sweat in mammals, water loss would assist in embryo temperature regulation against a higher ambient humidity.

Combined, it would seem that eggshells at expected temperatures and humidities (normal climate) have a regulatory effect on water loss, allowing normal embryo development within ‘normal’ ambient parameters, irrespective of eggshell thickness

At the climate change temperature of 32°C it seems as if water loss at different humidities remains the same and some ‘protective’ compensation is in effect, this ‘protective’ compensation effect broke down at 37°C. The standard deviations at 37°C were larger than at any other temperature (Figure 3.4.1). Climate change at extreme ambient temperatures is therefore likely to negatively affect the embryo, as water loss cannot be regulated by the shell anymore.

This was tested by plotting the %CVs of the humidities at each temperature irrespective of the magnitude of the humidity. These values are provided in Table 3.3 and plotted in Figure 3.4.2. The assumption is that compensatory and protective effects regarding water loss would result in low %CVs, but that the %CV will increase when this ability breaks down. This can clearly be seen in Figure 3.4.2 where the initial %CV at 12°C started to decrease from an amplitude of 14 %CV, to the lowest amplitude of 11.6 %CV at a temperature of about 27°C. The line stayed approximately horizontal for 15°C (between 17˚C and 32°C), where after it climbed quickly from about 30°C. This temperature width of 15°C was calculated by the ‘sum of two Lorentizian’ model (GraphPad Prism 2020). The model further predicted that the next amplitude would be at 42.6°C, with a %CV value of 60.8%. At that temperature, it is likely that water cannot escape through the shell any faster.

This model is very instructive as it indicates an optimum ambient incubation width of about 15°C – between 17°C and 32°C. Below and above this window, the eggshell seems to exceed its water loss regulatory abilities. Since no study has ever measured the three factors (various temperatures, various humidities, and different eggshell thicknesses) together in relation to water loss, this was the first indication of an optimal temperature/humidity width related to water loss. The lower temperatures seem to increase slowly in loss of water regulation, while at higher temperatures it seems to lose this ability much quicker. It would be very instructive to measure the eggs at even lower temperatures.

The apparent optimum temperature width of 15°C within which water loss seems best-regulated (Figure 3.4.2) at applicable humidities, is likely to be subjected to natural selection during climate change, assuming that the parent remains effective incubators at these increased temperatures. It is clear from Figure 3.4.1, that some eggshells were still able to regulate water loss at 37°C. Although a large number of eggs may be irretrievably affected by desiccation, there would be some that may manage higher temperatures better.

Different bird species breed at different latitudes and therefore different ambient conditions. Therefore, this model could be used to predict optimal temperature windows

based on water loss rates for different species, and be used in determining sensitivity to climate change.

4.5 Effect of eggshell thickness and pore density on water loss

As mentioned in Section 4.2, there were no significant differences in pore densities of the eggshells between the two islands (Figure. 3.2.2). Pore density in some bird eggshells increases with eggshell thickness; this is expected because thicker eggshells have to have longer pores, which in turn decrease the rate of water loss and overall effective pore area (Ar et al. 1974; Stein & Badyaev 2011). In this study however, pore density decreased with an increase in eggshell thickness (Figure 3.5.1) - therefore the opposite of what was found by others (Ar et al. 1974; Stein & Badyaev 2011). It should be kept in mind though, that the regression was non-significant ($p = 0.0759$), but only marginally so.

Since different female birds produce eggs that differ in pore densities, it is possible that incubation regimes are influenced by this factor. Water loss is higher at higher pore densities, with a concomitant quicker embryo development and hatching (Deeming 2008). Mechanisms that control pore formation are poorly understood, although it is thought that it is influenced by the number as well as the distribution of the palisade layer as well as the mammillary sites during egg formation (Section 1.6.1) (Ketta & Tůmová 2016). In Figure 1.2, the different layers of an African penguin eggshell were identified using a SEM showing an example of a pore. Unequal distribution of calcite columns and mammillae cause gaps in the eggshells, called pores. A previous study found evidence of a correlation between the number of mammillae and the pore density for other bird eggs (Tullet 1975), but this was not investigated during this study. Thus, attention needs to be given to the factors influencing the distribution and number of mammillae (Board & Scott 1980) in the African Penguin eggshell in future studies.

Since the eggs were collected freshly-laid (Section 2.1), and there was no significant difference in thickness, it can safely be assumed that the embryo development stages were equal between the islands. This information is important because the length of pores differ slightly at different developmental stages (Massaro & Davis 2004; Boersma et al. 2014; Vanstreels et al. 2019). The ages of the female penguins that laid the eggs are also unknown. The ages of the females can also affect pore density; as older females lay eggs with a higher pore density to enable a shorter incubation (Deeming 2008). Therefore, future studies could investigate how maternal characteristic affect egg water loss.

Water loss data acquired from incubating the eggs at different treatments (Table 1.1) were compared to pore density (Figure. 3.5.2). None of the slopes for each different temperature and humidity treatment were significant, thus indicating that pore density had no effect on water loss. This was unexpected in that the shells with the highest pore density (68 pores/cm^2) had double the pore density of the shells with the lowest pore density (33 pores/cm^2). This contradicted the expectation (based on the work by (Ar, et al. 1974; Stein & Badyaev 2011)) that an increase in pore density would be commensurate

with an increase in water loss. Why pore density did not influence water loss in the African Penguin shell is not clear, nor is this relationship known for other penguin eggs.

4.6 Water loss for the same humidity at different temperatures according to eggshell thickness

The influence of humidity on water loss according to eggshell thickness was shown in Figure 3.3.1. The effect of humidity on water loss was noticeable; therefore, the effect of different temperatures at the same humidity was regressed against eggshell thickness and the results are presented in Figure 3.6.1. All of the regression slopes were non-significant. Water loss was not influenced by eggshell thickness irrespective of temperatures for each humidity treatment (Figure 3.6.1). This contradicts the expectation that with the increase in eggshell thickness, water loss will decrease, as has already been concluded in Section 3.3. However, there were highly significant differences between the different temperature regression elevations with the increase in temperature. The amount of water loss also increased with an increase in temperature and decrease in humidity. An increase in temperature therefore, at each humidity, leads towards increased water loss, although this loss is not influenced by eggshell thickness.

The water loss at the highest temperature of each humidity treatment was the greatest at each humidity treatment, irrespective of the temperature itself (Figure 3.6.1). Climate change predicts higher temperatures. Therefore, even if humidity remains the same, an increase in temperature would result in greater water loss. The influence on water loss from bird eggs due to climate change has been previously recognized by others (Järvinen 1994; Crick 2004; Potti 2008) but not measured by any.

The African Penguin is able to modify behaviourally egg temperature rather than humidity when incubating their eggs (Wilson & Gremillet 1996). However, with global warming, this ability by the incubating penguin to regulate egg temperature will become less effective and an even greater challenge. Even though penguins co-parent during incubation, the effects of lower ambient humidity combined with increased temperature may cause increased desiccation and hence affect embryo development (Nakage et al. 2003).

Natural African Penguin nests are exposed to high average daily temperatures of up to 30˚C, but extreme temperatures can reach 40˚C (Griffin 2005). Temperatures in artificial nests were constantly higher than natural burrow temperatures, and they remained higher for longer periods of time, especially the fibreglass artificial nests (Lei et al. 2014).

There is very little other evidence that climate change will directly affect penguins, apart from more distant food stocks (Alheit et al. 2012; Sherley, et al. 2013). The reason for the lack of direct impact of climate change may be that long-term biological monitoring data is lacking. Thus, it is difficult to determine the causes of recently detected changes in the African Penguin population numbers, or even other large-scale marine ecosystem changes (Boersma 2008). However, for my study, seasonal changes can definitely be considered to having an effect on embryo development due to high water loss, especially

when eggs are left unattended during summer months when overheating is a likelihood (Stein & Badyaev 2011).

4.7 Water loss between clean (washed) and dirty (EEM) eggs

There are different types of pores in bird eggshells. Except for the definite ‘saucer’ shaped pores found in penguin eggs (Boersma 2014), there was no information in published articles on pore types of African Penguin eggs. Due to the insufficient information, a discussion on the phylogenetic implications of possible types of pore and their effects on water loss cannot be constructed.

Nevertheless, the eggs that were collected were covered in a sticky, oily EEM, presumably derived from the parents and surrounding nest area. I assumed that EEM would cause blockage of pores, resulting in decreased water loss. On the other hand, it can also be expected that water loss can increase with these EEM-covered eggs. The lumen of the pore canals exchanges water vapour with the nest microenvironment (Board & Scott 1980) thus providing a continuous link for water vapour to travel through. It is safe to conclude that eggshell and pore formation is a complex system that acts as a mediating boundary for embryo formation, including the regulation of water loss.

In Section 3.7, I measured and compared eggshell thickness of clean and EEM-covered eggshells, and found that EEM-covered shells were significantly thicker ($p = 0.0252$), as can be expected. I then measured the water loss according to thickness. As for the previous findings in Section 3.3 and discussed in Section 4.3, eggshell thickness did not have an effect on water loss. The slopes were effectively horizontal, with no differences in slopes. The horizontal differences in slopes were also not different, although only marginally so ($p = 0.1035$). This difference was further tested with a Mann-Whiney test, which showed a clear difference in water loss between clean and EEM-covered eggs. However, what was not expected was that the EEM-covered eggs lost water at a higher rate than the cleaned shells. This is interesting because it means that the water loss determined during this study could have been even higher than they already were because they were washed. The only explanation may be that EEM acts like a ‘wick’, actively assisting water vapour transfer, but it is not a very convincing argument, given the oily and sticky nature of the EEM.

Whatever the reasons may be, it implies that actual water loss from EEM-covered eggs could actually be higher than measured for ‘clean’ eggshells, exacerbating the threat of climate change on embryo development. The effect of EEM on water loss from eggs needs further investigation.

4.8 Water loss comparison between chicken and African Penguin eggshells

The comparison of data and findings of water loss between the African Penguin with another bird species would be valuable and insightful. The only comparable data available is from Veldsman et al. (2020). As expected, the chicken eggshells were thinner and the African Penguin eggs were thicker (Figure 3.8). Water loss was also higher through the thinner eggshells (chicken) than through the thicker eggshells (African Penguin). What is immediately apparent though was that water loss decreased significantly with an increase in thickness of the chicken egg, while it remained constant, and non-significant through the African Penguin eggshells. The same methods and incubator was used; so other factors can be excluded. What did correspond between the two species was that for each comparison more water was lost at higher temperatures (Figure 3.8). How chicken eggshells and penguin eggshells affect water loss is therefore quite different (under the conditions that I used), and something very much unexpected.

There may be factors in play that were not apparent at the beginning of the study.

- The elevation above sea level where the datasets for chicken eggs and penguin eggs were generated was the same and done in Potchefstroom. However, the African Penguin breeds at sea level. The difference in air and partial pressures between where they were laid and where they were measured is possibly a factor. The altitudinal difference is about 1300 metres with a calculated altitudinal air pressure of 80.7 kPa at Potchefstroom, while the pressure at sea level would be 101 kPa. At which altitude the chicken eggs were laid is not known, but they are likely to have been sourced locally, at higher altitudes. Even so, that a difference in altitude would result in no effect of eggshell thickness on water loss is remote. However, it would be advisable that similar studies be carried out at altitudes where the eggs were laid, or at least in pressure-regulated incubators. This would mean that water loss should be studied under controlled temperature, humidity, and air pressure, while taking eggshell thickness and EEM into account.
- In addition to the above argument would be the possibility that bird eggs would be adapted to the altitudinal range of the species. Altitudinal adaptation and preference by different species was recognised as a factor when species would have to redistribute due to climate change (Deeming 2008; Stein & Badyaev 2011). A whole range of studies on water loss dynamics of eggshells can therefore be conducted on inter- and intra-species altitudinal adaptation.
- There may be a difference in how marine, freshwater, and terrestrial birds construct their eggshell *vis a vis* water loss regulatory abilities. The sources of calcium may play a role. I could find no mention or study of such a factor, and it would be very interesting to compare aquatic and terrestrial bird eggs of about the same size and altitude.

4.9 Contamination

Birds affected by pollution may not only lay thinner eggs (Bouwman et al. 2015), but they may not breed at all to spend more energy in detoxifying themselves (Tartu et al. 2014). Ratcliffe (1970) was instrumental in proving the connection between the spraying DDT and eggshell thinning leading to reproductive failure. As discussed in Section 1.7, persistent organic pollutants change the function associated with oogenesis, like changes in absorption and secretion of bicarbonate, which ends up changing pore density in the eggshells (Stein & Badyaev 2011). Many bird species have been affected by DDT. Heath et al. (1969) connected it to population decline. The reason for a declining bird population is of a critical level where eggshell thinning occurs and the incubating parents cause damage to the eggs, causing water loss. Bouwman et al. (2015) found high residues of POPs linking it to a 38% (RIS) and 40% (BIS) eggshell thinning of African Penguin eggshells.

It is important to monitor contaminants in biota and especially their influence at a regional scale. This will lead to early detection of any contaminant concentration and its adverse effects (Bunck et al. 1985). More importantly, marine environmental policy can be informed about important implications for specific species and ocean health. There is very little large-scale pollutant monitoring that includes seabirds even though they are considered effective monitors. In the case of the African Penguin however, although thinner eggs were associated with increased concentrations of POPs, the thinner eggshells did not lose more water than thicker eggshells. The case that I made that eggshells that are thinner, presumably by pollution, would lose more water and their embryos would be greater risk of effects of desiccation, can therefore not be supported by current data.

4.10 Population

The great concern about the already endangered African Penguins prompted conservation and research bodies to look for causes and solutions (Shannon & Crawford 1999). My study is a new approach for the African Penguin and provides the first indication that global warming may move beyond the optimal breeding window for temperature (Figure 3.4.2). The protection and conservation of the African Penguin depend also on things like the availability and location of food sources. There is an eastward shift in pelagic resources that may be linked to climate change. Since nothing can be done to this external force, conservation measures should be put in place to relieve manageable stressors for the African Penguin (Crawford et al. 1995).

Breeding success of the African Penguin in artificial nests should be monitored to ensure acceptable temperatures (Ropert-Coudert et al. 2004) (Connan, et al, 2016). If not, current declining population trends may continue. My study indicated an optimal temperature window of between 17°C and 32°C (Section 4.4). The temperature of the incubating adults is also important because it would influence the incubating penguin's behaviour (Grant 1982). This can be further studied by looking at the thermoregulatory behaviour of penguins (Šálek & Zárybnická 2015). Their behaviour will indicate if they are heat-

stressed. For instance, they might rather spend their time in the water or in shade rather than incubating their eggs when it is too hot (Green & Pichegru 2014). Weather stations monitor the daily maximum and minimum temperatures and humidity (Ropert-Coudert et al. 2004). This data may provide an indication of when the temperature window that I identified might be exceeded, as well as actual changes in humidity, rather than the humidities I assumed.

CHAPTER 5: CONCLUSSION

5.1: Summary

The African Penguin is an Endangered species occurring along the Indian and South Atlantic coasts of South Africa. There are many suspected causes for the precipitous decline in their numbers such as the movement of food further away from their onshore breeding colonies, and pollution. A previous study found eggshell thinning associated with increased concentrations of POPs in two breeding colonies: Robben Island and Bird Island (Bouwman et al. 2015). Since global climate change is happening with an expected increase in temperatures, the combination of thinner shells and higher temperatures may cause accelerated water loss through the shells, thereby possibly harming the embryo.

To test this scenario, a method had to be developed that made it possible to measure water loss across eggshells under controlled temperature and humidity treatments. This I did in a previous study where I converted a chicken egg incubator, and successfully tested the effectiveness using chicken eggshells. Thinner eggs lost water faster than thicker shells at higher temperatures, moderated by humidity (Veldsman et al. 2020).

Having developed this system, I used the eggshells from the eggs collected by the Bouwman et al. (2015) study to measure the effects of temperature and humidity on water loss. The cleaned eggshells from the two islands were equivalent in thickness, pore densities, and water loss characteristics, so I combined the two sets of ten eggs into one pool of twenty for further testing. I chose temperature and humidity treatment based on current climate data, and selected two higher temperatures (32°C and 37°C) and commensurate humidity settings to simulate future climate change scenarios.

African Penguin eggshells lost water faster at higher temperatures, and less water was lost at higher relative humidities irrespective of temperature, as was the case for chicken eggshells. However, and unexpectedly, eggshell thickness did not play a role. All eggshell thicknesses tested lost water at the same rate, which was not the case for chicken eggshells, as tested earlier. Thinner and thicker eggs lost water at the same rate at all temperature and humidity treatments. Water loss across the eggshells was therefore regulated by the shells, irrespective of their thickness, and, as it turned out, also their pore densities that only marginally reduced with thicker eggshells.

Looking at the data (Figure 3.4.1), I noticed that the standard deviations of the scatterplots increased at higher temperatures. Plotting the %CVs of the water loss at each relative humidity against their respective temperatures showed that the regulatory effect (low %CVs) was lost at around 32°C, and increased towards 37°C (Figure 3.4.2). The fitted model also suggested that at less than 21°C, water loss regulation would also decrease. An optimal 15°C temperature window for effective water loss was indicated by the model.

Since no study has ever measured the three factors (temperature, humidity, and eggshell thickness) in combination regarding water loss, this was the first indication of an optimal temperature/humidity width related to water loss. The lower temperatures seem to increase slowly in loss of water regulation, while at higher temperatures it seems to lose this ability much quicker. Not all shells responded in this way, suggesting a variability for natural selection to act upon. Such a temperature window may therefore be able to shift with climate change, assuming that parents remain effective incubators at higher temperatures.

One factor remained to be tested and that was the effect of eggshell extraneous materials (EEM), an oily and sticky dirt-covering, on water loss. Comparing 'clean' eggshells (that were also thinner due to the EEM being removed) with the 'dirty' eggshells unexpectedly showed a significant faster water loss for the EEM covered eggshells. I cannot adequately explain this, as 'wicking' of water though an oily layer is counter intuitive. This issue requires further testing.

An aspect to keep in mind was that the tests were carried out at a lower air pressure (80.7 kPa) at an altitude of 1300 m above sea level with an air pressure of 101 kPa. Differences in pressure may affect the amounts of water loss, but cannot explain the lack of effect of thickness. Bird eggs are likely to be adapted to the preferred altitudes of the respective species, suggesting an interesting research avenue. It may also be that terrestrial, and marine- and freshwater species construct their eggs differently, another avenue of investigation.

Therefore, although this study started off with a concern raised by thinner associated with increase POPs concentrations in the eggs, I could find no water loss effect related to this parameter. It does not negate that thinner eggs are able to withstand other forces such as pressure exerted by their incubating parents, but, in the way I measured water loss, thinner eggs associated with pollution did not seem to be negatively affected. Thinner eggs are also more susceptible to infection.

The effect of water loss on population size remains to be determined. However, I showed that increased temperatures are likely to increase water loss rates, thereby placing metabolic stress on the developing embryo. The compensatory abilities of the embryo might be exceeded at such higher temperatures. Climate change therefore, will add pressure on African Penguins though accelerated water loss, adding to the pressures such as pollution and food depletion and movement.

5.2: Considerations for future studies

There were some knowledge gaps identified in this study, which could open important perspectives. For instance

- It is important to look at the thermo-neutral zone for the African Penguin. By studying the thermoregulatory behaviour of the penguins, it should be possible to determine when they are heat-stressed (Šálek & Zárybnická 2015). This is much-needed information since it is an indicator of behaviour on how they conserve water, dissipate energy, and reduce thermal stress (including during incubation), much the same as I determined a temperature window where water loss regulation is effective.

- Thinning of the eggshell beyond what is expected for egg size increase the chances of infection (Heming & Marini 2013). Thus, a greater pore number compensates for increased fungal or bacterial infection.

- Further studies are needed on the microscopic eggshell structure of penguins, the result of blocked pores and EEM, and possible effect of evaporative cooling when wet parent incubate eggs (Portugal et al. 2010).

- Shell composition is also very important and needs further investigation like analysis of calcium and protein composition due to its influence on the function of the eggshell (Ketta & Tůmová 2016). Knowledge on eggshell composition and structure will provide additional information and understanding of the determinants of porosity and even possible aspects of shell strength. Specific attention should be given to the inner part of the palisade layer. Hopefully, this would lead to better knowledge on the adaptability of penguin eggshells for incubation in extreme environments (Lomholt 1976) (Rahn & Ar 1974).

- A study found that there is evidence of the correlation between the number of mammillae and pore density (Tullet 1975), but it was not looked at in this study. Thus, attention can be given to the factors influencing the distribution and number of mammillae (Board & Scott 1980).

- The penguin eggshell was large enough in terms of length and thickness to successfully cut them and glue them onto 4 ml vials. For future studies, using smaller eggs may be difficult and too fragile to handle and fit on vial caps. Smaller vessels should be used.

- Since the African Penguin can also be found on the coastline of Namibia, this study could be repeated to see if the relationships hold as I tested.

- Future research should also consider the temperature the eggshell itself reaches, not only the ambient temperature (Boulton & Cassey 2014).

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