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The effect of season, laying order and female age on the microstructure and properties of African penguin (Spheniscus demersus) eggshells

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Article

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Abstract

The African penguin (*Spheniscus demersus*) is a critically endangered species whose long-term conservation relies on ex situ breeding programs. Despite the importance of studies on the biology of this species and reproduction in captivity, little is known about the eggshell microstructure of this species and the factors influencing its variation. This study investigated the effects of female identity, age, laying order, and season on eggshell microstructure, thickness, mechanical properties and their interrelationships. A total of 318 eggs were collected from the breeding colony at Wrocław Zoo of which 175 eggs from 35 females met the criteria for detailed analyses. Most microstructural traits exhibited high repeatability within females, indicating substantial individual variation in eggshell characteristics. Laying order significantly affected the mammillary layer, with second eggs having a greater density of mammillary knobs ($p < 0.001$) but smaller individual knob size ($p < 0.001$) than first eggs. In contrast, seasonal differences were negligible and, although occasionally statistically significant, were not considered biologically meaningful. Female age influenced several eggshell characteristics: young females produced eggs with thinner eggshells ($p < 0.001$), mainly due to a thinner palisade layer, whereas older females maintained eggshell thickness despite slight changes in mammillary layer morphology. Eggshell strength and elastic deformation were primarily associated with overall eggshell thickness and egg shape rather than with individual microstructural traits. These findings demonstrate that eggshell characteristics in African penguins are shaped predominantly by individual, innerclutch and age-related factors, whereas seasonal conditions have little biological relevance.

Introduction

The African penguin (*Spheniscus demersus*) is estimated to become extinct in the wild within the next few years. Its population has been declining steadily since the mid-20th century, and the most recent study published in 2024 demonstrated that the breeding population had decreased by nearly 78% over the past 30 years [1]. This decline, together with an estimated population size of approximately 9,900 breeding pairs, formed the basis for classifying the species as Critically Endangered. Historically, the decline of African penguin populations was primarily attributed to guano harvesting and egg collection [2]. Currently, overfishing is considered the principal driver of population decline, as it not only reduces breeding success but also increases adult mortality. Between 2004 and 2011, following the collapse of the populations of their primary prey species, a significant increase in adult mortality was observed; approximately 95% of the birds that attempted to breed during the first year of the study died as a result of food scarcity over the subsequent eight-year period [3]. At present, the prospects for reversing this population decline remain uncertain. Although numerous conservation measures have been implemented, their scope is limited and insufficient to halt the ongoing decline. Under these circumstances, zoological gardens represent a genuine opportunity to safeguard the species from complete extinction. However, to fulfil this role effectively, a thorough understanding of the species' biology, particularly its reproductive biology, is essential. African penguins accustomed to human presence provide valuable opportunities for such research, including the development of semen collection and cryopreservation techniques [4,5]. Genetic biobanking may help mitigate the loss of genetic diversity within the population. The procedures developed using African penguins maintained in zoological collections may subsequently be successfully applied to wild populations [6,7].

African penguins are maintained in the collections of approximately 20% of zoological institutions accredited by the Association of Zoos and Aquariums [8]. In total, around 3,700 individuals are housed in zoological collections worldwide. The three largest colonies, each comprising more than 100 individuals, are maintained at Amsterdam Royal Zoo, the Maryland Zoo in Baltimore, and Wrocław Zoo [9]. Zoological institutions systematically collect and share data on parameters such as disease incidence, mortality, and treatment protocols [8]. These data have shown that aspergillosis is the leading cause of mortality in captive African penguins and that mortality rates increase after 15 years of age. In females, approximately 3% of deaths are associated with reproductive disorders, including dystocia (egg binding), egg yolk peritonitis, and salpingitis [8]. One of the earliest clinical signs of reproductive tract dysfunction may be abnormalities in egg appearance, such as eggshell deformities. Therefore, monitoring egg characteristics may provide a simple and non-invasive approach for assessing the reproductive health of females. Eggshell abnormalities have been reported during *Mycoplasma* spp. infections [10] as well as in salpingitis caused by various factors [11]. Nutritional deficiencies also affect eggshell appearance, eggshell quality, and female health, as demonstrated by the reported case of osteodystrophy in a captive African penguin [12]. Under natural conditions, females have been observed to selectively increase their consumption of molluscs, which provide the calcium required for eggshell formation [13]. Because the nutritional requirements of females change not only during the moulting period, as in males, but also throughout the reproductive cycle, adjusting the diet to the individual needs of each bird is challenging. Unlike flamingos, African penguins do not exhibit such a high degree of breeding synchrony, which would facilitate better alignment of current feeding programmes with the physiological status of the birds. Eggshell quality is particularly important in African penguins because they incubate their eggs on hard substrates composed of stones and gravel. Moreover, these birds are relatively heavy, reflecting adaptations to their aquatic lifestyle. Consequently, their eggshells must withstand greater mechanical loads than those of other bird species of comparable body size [14]. Despite this, studies on penguin eggs remain limited. To date, the structure and properties of African penguin eggshells have not been investigated. The closest related species for which such analyses have been conducted, albeit to a limited extent, is the Magellanic penguin (*Spheniscus magellanicus*), in which weak predictors of eggshell thickness and porosity were identified in relation to female age, egg-laying date, and nest type [15]. In contrast, studies on the Yellow-eyed penguin (*Megadyptes antipodes*) demonstrated differences in the porosity of sequentially laid eggs [16].

The aim of this study was to investigate the factors affecting the quality and properties of African penguin eggshells, with particular emphasis on their microstructure. We hypothesized the existence of inter-individual variation in eggshell microstructure and sought to quantify it. Furthermore, by examining the effects of environmental factors, such as weather conditions prevailing during different seasons (summer and winter), as well as physiological factors (egg laying order within the clutch and female age), we aimed to determine their influence on the reproductive potential of this species under captive conditions. Investigating the relationships between eggshell strength, elasticity and the characteristics of its microstructure enabled us to identify which microstructural features are crucial for maintaining high eggshell quality. The findings of this study may contribute to the conservation of this critically endangered species, the monitoring of the welfare of individuals maintained in captivity, and broader research on the reproductive biology of birds.

Results

Mammillary layer

After accounting for the individual effect (female ID), the number of mammillary knobs per mm^2 was not associated with season but was significantly related to laying order within the clutch. Second-laid eggs contained slightly more mammillary knobs (0.143 knobs per mm^2) than first-laid eggs (Fig. 1). For the percentage of the mammillary layer covered by knobs (knob coverage), neither season nor laying order had a statistically significant effect, although the effect of laying order approached the significance threshold. Another statistical model showed that season had no effect on mammillary knob size, whereas laying order significantly influenced this trait. Mammillary knobs in second-laid eggs were 14.1% smaller than those in first-laid eggs (Fig. 2). This finding suggests that the greater number of mammillary knobs observed in second-laid eggs resulted from a reduction in the size of individual knobs, without affecting the overall percentage of the mammillary layer covered by knobs. A similar pattern was observed in the analysis of knob area performed using the trained AI-based script, according to which mammillary knobs in second-laid eggs were 10.86% smaller than those in first-laid eggs. In addition, this analysis indicated that eggs laid during winter had mammillary knobs that were 3.05% larger than those laid during summer. Although this difference was statistically significant, we consider it to be biologically negligible (Table 1). Differences in mammillary knob size between first- and second-laid eggs were also reflected in other morphometric parameters: ESD was 5.44% lower in second-laid eggs, also F_{min} and F_{max} were reduced by 5.54% and 5.73%, respectively. However, these differences did not substantially affect knob shape, as reflected by the FR, which was only 1.01% higher in second-laid eggs. Although this effect was statistically significant, its magnitude was considered biologically negligible. Although the season had statistically significant effects on several morphometric parameters, the observed differences were too small to be considered biologically meaningful, amounting to 1.41% for ESD, 1.16% for F_{min}, and 1.41% for F_{max}. No statistically significant effects of either laying order or season were detected for FR (Table S1). The analyses revealed very high between-female variation in the number of mammillary knobs per mm^2 and, consequently, low variation among eggs laid by the same female (ICC = 0.92, indicating that 92% of the observed variation was attributable to differences among females). A somewhat lower, although still high, level of between-female variation was observed for knob coverage (ICC = 0.76). Both manually measured by researcher knob areas exhibited low between-female variation (ICC = 0.14) and the AI-based script (ICC = 0.06) showed low between-female variation. Low between-female variation was also observed for F_{min} (ICC = 0.08), F_{max} (ICC = 0.12), ESD (ICC = 0.12), and FR (ICC = 0.02).

Table 1
Variation in the microstructure of the mammillary layer according to the egg-laying period (season) and laying order within the clutch. The analyses were performed using generalized linear mixed-effects models (GLMMs). Depending on the distribution of the response variable, either *t*- or *z*-statistics were calculated. Values marked with an asterisk (*) represent *z*-statistics, whereas all remaining values correspond to *t*-statistics.

Analyze	Fixed effect	Estimate (β)	SE	t or z value*	p value
Knobs/ mm^2	Intercept	4.175	0.039	106.61	< 0.001
	The order of egg in clutch (second egg)	0.143	0.013	11.11	< 0.001
	Laying period (winter)	-0.020	0.016	-1.29	0.199
Knobs area %	Intercept	0.746	0.029	25.96*	< 0.001
	The order of egg in clutch (second egg)	0.026	0.014	1.85*	0.064
	Laying period (winter)	0.021	0.017	1.29*	0.198
Knob area μm_2 measured by researcher	Intercept	9.151	0.030	42.95	< 0.001
	The order of egg in clutch (second egg)	-1.152	0.006	-24.51	< 0.001
	Laying period (winter)	0.010	0.008	1.35	0.177
Knob area μm_2 measured by AI script	Intercept	8.994	0.012	782.81	< 0.001
	The order of egg in clutch (second egg)	-0.115	0.003	-36.74	< 0.001
	Laying period (winter)	0.030	0.004	8.08	< 0.001

Layers thickness

According to the models, neither laying order within the clutch nor season had a significant effect on overall eggshell thickness. When the proportional contribution of each eggshell layer to the total shell thickness was analysed, the models likewise showed no significant effects of either season or laying order. The only exception was the palisade layer, which was 1.5% thicker in eggs laid during winter. However, although this difference was statistically significant, its magnitude was considered biologically negligible (Table S2). Intraclass correlation indicated fair between-female variation in overall eggshell thickness (ICC = 0.43). Similar levels of between-female variation were observed for mammillary layer thickness (ICC = 0.43) and its proportional contribution to total shell thickness (ICC = 0.27), as well as for palisade layer thickness (ICC = 0.48) and its proportional contribution (ICC = 0.22). The crystalline layer exhibited the lowest between-female variation in absolute thickness (ICC = 0.07) and proportional contribution variation (ICC = 0.24).

Eggshell durability and elastic deformation

Neither season nor laying order affected eggshell strength. The model indicated fair between-female variation in this parameter (ICC = 0.56). Eggs laid during winter were slightly less elastic (by 0.04 mm), but this effect was not biologically significant. High between-female variation was observed for this parameter (ICC = 0.70). External egg parameters (length, width, length-to-width ratio, and total eggshell thickness) explained 50.45% of the observed variation in eggshell strength; eggs that were wider and shorter had more durable shells. Also shell thickness had a positive effect on strength. In the case of eggshell elastic deformation, these parameters explained 49.08% of the observed variation, none of the isolated parameters had a biologically significant effect. The number of mammillary knobs per mm² and the knobs coverage explained 16.96% of the observed variation in eggshell strength and 5.65% of the variation in elastic deformation. None of these isolated parameters had a biologically significant effect. Eggshell thickness and the thickness of its individual layers explained 35.76% of the observed variation in eggshell strength, of which only a greater palisade layer thickness influences significantly. For elastic deformation, the thickness of the individual eggshell layers explained 40.71% of the observed variation, with again the effect of the isolated layer did not have a biologically significant impact. Pearson's correlation analysis showed that the proportional contribution of the crystalline layer was significantly negatively, but weakly, correlated with both eggshell strength and eggshell elasticity.

Female age and microstructure

The youngest females had a higher number of mammillary knobs per mm² ($p < 0.001$; DF = 2; H = 18.23) than females from the other two age groups, which consequently resulted in the highest coverage of the mammillary knobs in this group ($p < 0.001$; DF = 2; H = 51.80). The mammillary knobs of these females were also slightly smaller than those of the remaining age groups ($p < 0.001$; DF = 2; H = 41.77) (Table 2). However, when the trained AI-based application was used to measure mammillary knob area, the smallest knobs were found in females from the middle age group, followed by the youngest females, whereas the largest knobs occurred in the oldest females ($p < 0.001$; DF = 2; H = 39.05). Although Dunn's post hoc test showed differences between all age groups, an examination of the median values indicated that these differences were small, and therefore the biological effect is questionable. A similar pattern was observed for the remaining mammillary knob parameters (F_{min}, F_{max}, ESD, FD) where the *p*-values indicated statistically significant differences, but the biological effect was minimal (Table S3). For total eggshell thickness, the differences were both statistically and biologically significant ($p < 0.001$; DF = 2; H = 33.81). Younger females laid eggs with thinner eggshells than females aged 5–14 years ($p < 0.001$; z = 5.339) and females older than 15 years ($p < 0.001$; z = 5.183). No difference was found between females aged 5–14 years and those older than 15 years ($p = 0.480$; z = 1.406) (Fig. 3). This difference resulted from the thickness of the palisade layer ($p < 0.001$; DF = 2; F = 20.38), with younger females having this layer thinner significantly than the other age groups. Although, similarly to the previous analysis, significant differences in mammillary layer thickness were found among the age groups ($p = 0.015$; DF = 2; F = 4.31), both the mean values and the relatively low F-value do not indicate a biologically significant effect. No differences in crystalline layer thickness were found among the age groups ($p = 0.730$; DF = 2; F = 0.31). Although the analyses revealed statistically significant differences in the percentage contribution of the individual eggshell layers (mammillary layer: $p = 0.037$; DF = 2; F = 3.36; palisade layer: $p = 0.001$; DF = 2; H = 13.52; crystalline layer: $p = 0.033$; DF = 2; H = 6.83), these differences were sufficiently small that they were not considered biologically significant.

Table 2
Microstructure of the mammillary layer in different age groups of African penguins (young females: 3–4 years; females at peak reproductive period: 5–14 years; older females: >15 years)

Microstructure parameter	Female age group	Median	z value
Knobs per mm^2	3–4 years	82.85	2.92
	5–14 years	79.76	0.45
	> 15 years	71.09	-3.57
Knobs area (%)	3–4 years	70.72	4.06
	5–14 years	69.62	1.83
	> 15 years	66.48	-6.52
Knob area μm_2 (measured by researcher)	3–4 years	7301	-4.48
	5–14 years	7562	-0.65
	> 15 years	8019	5.32
Knob area μm_2 (measured by AI-based script)	3–4 years	6210	-2.16
	5–14 years	6325	5.78
	> 15 years	6122	-5.44

Discussion

Variation in avian eggshells has been well documented with respect to differences among reproductive strategies [19], taxa [20], and species [21]. In contrast, intraspecific variation is less well understood and may result from long-term selection processes associated with domestication [22] or from nutrition [23]. Age may also influence eggshell structure [24], as a consequence of changes in ovarian function [25]. It should be noted, however, that studies investigating the effects of nutrition and aging on eggshell structure have been conducted primarily in poultry. Although the general structure of the eggshell and the physiology of reproduction are similar across all avian species, poultry represent a specific research model that cannot be fully extrapolated to non-domesticated birds. Poultry lay a greater number of eggs, and their laying cycle is prolonged, whereas in non-domesticated birds, clutch size and breeding frequency depend on factors such as latitude [26] and longevity [27]. African penguins lay only two eggs per clutch; therefore, an individual reproductive cycle is less demanding than in poultry. One of the aims of our study was to determine whether, despite such low fecundity, eggs within the same clutch differ in eggshell microstructure. In second-laid eggs, the number of mammillary knobs was higher as a result of a smaller mammillary knob area; however, this did not significantly affect knob coverage of the mammillary layer. The mammillary layer is responsible for supplying calcium to the developing embryo, as reflected in the structural differences observed between altricial and precocial species, which differ in their calcium requirements [19]. Such differences are also present within the same reproductive strategy when the degree of skeletal ossification of the chick at hatching is taken into account [20]. African penguins exhibit asynchronous hatching, resulting in age differences between siblings. Consequently, the younger chick must compete with its larger and stronger sibling for resources. It is well established that females can manipulate parental investment through adjustments in yolk hormone levels, antioxidant concentrations, and fatty acid profiles, thereby influencing offspring phenotype [28]. Studies suggest that this may increase the competitiveness of younger chicks within the brood. However no study has been conducted to examine variation in eggshell microstructure within a clutch in non-domesticated birds. Considering the function of the mammillary layer and the within-clutch variation observed in the present study, we hypothesize that eggshell microstructure may influence the efficiency or rate at which the developing embryo utilizes the calcium stored in this layer. This hypothesis, however, requires further verification supported by additional studies.

Contrary to our expectations, we found no evidence that season affects African penguin eggshell characteristics. This hypothesis was based on the assumption that environmental conditions, including solar radiation and day length (and consequently vitamin D production, which plays a key role in calcium–phosphorus metabolism) would influence eggshell formation. Also temperature differences between summer and winter in the natural range of African penguins are not as pronounced as those experienced in central Europe. Nevertheless, this does not appear to have affected the reproduction of this species under captive conditions. In the wild, African penguins are capable of breeding throughout the year, although an increased number of breeding pairs is observed during certain periods [29]. Breeding synchrony was greater in colonies with higher nest densities, which, according to the authors, suggests that breeding phenology is influenced more by social interactions than by seasonal effects. In our study, we observed two peaks in egg laying, one during winter and the other during summer; however, eggs were laid throughout the entire year. This would support the conclusion that the reproductive period of this species is not dependent on weather conditions or day length, but rather on other factors that are not yet fully understood.

Our results show that certain features of eggshell microstructure are highly conserved (mammillary knob shape and, to some extent, mammillary knob area), whereas others (the number of mammillary knobs and mammillary knob coverage) are influenced by various factors. We observed inter-individual variation in eggshell structure and, to a lesser extent, variation within clutches. Although our analyses identified the effects of multiple factors on eggshell microstructural variation, in most cases these effects were sufficiently small that they cannot be considered biologically significant. The factors that proved to be important for many microstructural parameters was the previously discussed laying order within the clutch and female age. Because the number of females was too small to obtain a representative sample for each individual year of age, we divided the females into three age categories reflecting physiological differences among these groups. The results indicate that, despite their advanced age, African penguins retain their reproductive capacity (defined in the present study as normal eggshell microstructure). It should be noted, however, that we did not assess egg production itself. During egg collection and selection for the analyses, we observed that it was not possible to obtain as many clutches from older females (including two-egg clutches) as from younger females. In addition, older females tended to lay eggs with a slightly lower number of mammillary knobs, which resulted in a lower percentage of mammillary knob coverage. However, these females maintained normal eggshell thickness, which, according to our analyses, together with egg shape, determines eggshell strength. Females from the youngest age group had thinner eggshells, which resulted from a thinner palisade layer. Young birds may be less efficient at transporting calcium resources to the eggshell or, which we consider more likely, despite receiving regular care and feeding, they may be less efficient at foraging than older birds. Based on observations made by the bird keepers, we infer that this reduced foraging efficiency may be result from the challenges associated with their first breeding attempts, including establishing and maintaining a nest and acquiring and retaining a mate. For younger birds, the appropriate allocation of energetic and time resources between reproduction and functioning within the colony may represent a greater challenge. There is no evidence in the literature that young birds tend to produce eggs with thinner eggshells. Young birds lay smaller eggs, but not eggs with thinner shells [30]. Within a species, eggshell thickness is influenced by factors such as environmental pollution [31,32], the availability of calcium sources [33] and laying order within the clutch, which is associated with the depletion of stored calcium reserves [34]. Because all birds in the present study had access to the same food source and lived under the same environmental conditions, environmental factors do not appear to account for the observed differences. This indicates the need to investigate the behaviour of African penguins from different age categories maintained in zoological gardens. To date, only two studies have addressed this issue. They showed that the frequency of behaviours such as object play is higher in juveniles, whereas self-preening and social preening occur less frequently in this age group [35]. Other studies have shown that, under captive conditions, young African penguins begin breeding attempts at an earlier age; however, successful reproduction still requires experience [36].

In the present study, we also examined the effects of season, laying order within the clutch, eggshell microstructure, and egg shape on eggshell strength and elasticity. For the first two factors, we found no significant effects on the analysed parameters. For the number of mammillary knobs and palisade layer thickness, we found statistically significant, but very small, effects. Although the literature indicates that the palisade layer is responsible for eggshell strength [37], its effect is not particularly large. Eggshell strength and elasticity proved to result from the combined action of multiple factors, of which total eggshell thickness and egg dimensions were the most important. We reached similar conclusions in our previous study, in which we demonstrated that more spherical eggs have greater strength, resulting from a different distribution of forces during the application of a point load [38].

The microstructural analyses performed in the present study were highly time-consuming, which, in addition to the cost of imaging the samples, limits the feasibility of conducting similar studies. For this reason, we developed an application that automated part of the analyses by estimating the percentage coverage of the mammillary knobs and measuring the morphometric parameters of individual knobs. The development of the application required training the AI model to correctly identify the boundaries of individual mammillary knobs. The application substantially accelerated the analysis; however, the quality of its output was also verified by the researcher. The AI-based script tended to underestimate mammillary knob coverage when image regions were out of focus. Thus, the quality of the analysis performed by the script depends largely on image quality, which may be problematic when photographing spherical objects such as eggshells. The script is, however, capable of performing a much larger number of measurements and eliminates researcher bias, whereby the researcher may preferentially select the most conspicuous and easiest-to-measure structures within an image. The results obtained by the researcher (– 14.1%) and by the script (– 10.86%) for differences in mammillary knob size between first- and second-laid eggs were similar. Some discrepancies in the comparison of eggshell microstructure among different age categories require further investigation to determine their underlying causes. The script can likely be further improved, and we intend to continue its development.

The conducted research helped expand knowledge on the reproductive biology of African penguins kept in zoological gardens. Inter-individual, age-related, and within-clutch variation was demonstrated, whereas season had no effect on the evaluated parameters. We hypothesize that within-clutch variation may be driven by mechanisms aimed at equalizing the chances of younger chicks when competing for resources with their siblings. In turn, variation across age groups may result from a lack of breeding experience and lower resource acquisition efficiency in young birds; however, this hypothesis requires verification through behavioral studies. The physical strength and elasticity of the eggs were primarily determined by eggshell thickness and shape, and to a lesser extent by microstructural parameters.

Material and methods

Study flock and egg collecting

All eggs were collected from the African penguin colony maintained at Wrocław Zoological Garden. This colony consists of approximately 100–125 individuals, with fluctuations in numbers resulting from chick hatching and transfers of birds to other institutions. The exhibit comprises a 900 m² beach area and a pool with a surface area of nearly 1,000 m². The pool is 4.5 m deep and contains 2,460 m³ of water. The birds are provided with artificial nest boxes, which are inspected daily by animal care staff. Most laid eggs are removed, while the decision regarding which individuals are allowed to reproduce is made by the species coordinator. To prevent the laying of replacement clutches, which would increase the reproductive burden on females through additional egg production, dummy eggs are placed in the nests after the original eggs have been removed.

Over a period of 1.5 years (from August 2024 to February 2026), a total of 318 eggs were collected. Immediately after collection, the maximum egg length and width were measured using digital calipers. Eggs were collected within 24 hours of laying and stored at 7°C until analysis, preventing embryonic development. No eggs were incubated, and none contained a developed embryo that could affect eggshell microstructure. Eggshell strength (kg) and deformation, defined as eggshell deflection at the point of fracture (mm), were determined using a TA.XTplusC Texture Analyser (Stable Micro Systems, Surrey, England). During testing, eggs were positioned horizontally on the testing platform, corresponding to their natural orientation in the nest. From the collected material, 175 eggshells originating from 35 different females were selected for further analyses according to the following criteria: (1) absence of any signs of embryonic development within the egg; (2) availability of at least four eggs collected from the same female during different seasons of the year (winter: from the second half of November to the end of February; summer: from the second half of May to the first half of September); and (3) inclusion of females representing the widest possible age range (3–24 years). Females were assigned to one of three age categories: young females, shortly after reaching sexual maturity (3–4 years of age; n = 5); females at peak reproductive performance (5–14 years of age; n = 24); and ageing females (> 15 years of age; n = 6). The threshold for the ageing category was established based on published literature indicating a significant increase in mortality after this age [8]. The winter and summer periods were defined according to both photoperiod and ambient temperature. During the designated winter period, day length ranged from 8 to 11 hours, whereas during the summer period it ranged from 13.5 to 16.5 hours. Mean daily air temperatures averaged 2.5°C in winter and 18.5°C in summer.

Eggshell microstructure analyses

A fragment of approximately 1.5 cm² was collected from the central region of the lateral surface of each eggshell. The samples were then boiled for 7 minutes in a 5% NaOH solution to remove the eggshell membranes, which would prevent the evaluation of the microstructure of the inner (mammillary) eggshell layer. The prepared samples were dried at room temperature for 48 h, and then were coated with gold using a Sputter Coater S150B (Edwards/Scancoat Six, HHV Ltd., Crawley, UK) with a 300-s coating program. Those prepared samples were examined using a scanning electron microscope (SEM; EVO LS 15, Zeiss, Oberkochen, Germany). Three micrographs of the eggshell cross-section were obtained from each sample at ×100 magnification, allowing measurements of the thickness of the three eggshell layers: the inner mammillary layer, the middle palisade layer, and the outer crystalline layer (Fig. 4). Based on these measurements, the percentage contribution of each eggshell layer to the total shell thickness was calculated. Also four micrographs of the mammillary layer were acquired from each sample at ×70 magnification. These images were used to determine the number of mammillary knobs visible within the field of view and to measure the area of individual knobs (µm²), maximum Feret diameter F_{max} (Feret diameter with the greatest length), minimum Feret diameter F_{min} (Feret diameter with the shortest length), Feret ratio FR (an indicator of object elongation) (1), Equivalent Shape Diameter (ESD; an indicator of object circularity) (2), and the percentage of the surface area covered by mammillary knobs. Measurements of eggshell cross-sections, counts of mammillary knobs per mm^2, and measurements of their surface area were performed using Fiji (ImageJ 1.53n, Wayne Rasband, National Institutes of Health, USA). The remaining morphometric measurements (F_{max}, F_{min}, FR, ESD) together with an additional. independent measurement

of mammillary knob area, were obtained using a custom-written image analysis script. Each image processed by the script was manually verified to ensure the accuracy of the automated analysis.

$$FR = \frac{Feret\ min}{Feret\ max}$$

(1)

$$\mathrm{ESD} = \sqrt{\frac{4 * \mathrm{knob\ area}}{\pi}}$$

(2)

AI script written to image processing

The development of the AI model (download source from [17]) began with the preparation of a reference dataset consisting of 60 randomly selected images. Mammillary knob annotation was performed using the open-source software AnyLabeling, version 0.4.0 [18]. To optimize the training process and ensure adequate model generalization, a custom script was developed to automatically divide the images into smaller patches and randomly shuffle them. The resulting dataset was subsequently split into training and test/validation subsets. Rather than training the neural network architecture from scratch, a pretrained model was employed, allowing the algorithm to benefit from previously acquired knowledge of basic geometric structures and object shapes. The YOLOv11 Medium architecture was selected as the core object detection framework because it provides an optimal balance between computational efficiency and real-time detection accuracy. In addition, the framework incorporates integrated on-the-fly data augmentation techniques, enabling random modifications of the training images, including variations in colour and contrast, geometric transformations (rotations and horizontal flipping), image scaling, and changes in input resolution. These augmentations increased the diversity of the training dataset and reduced the risk of overfitting. Upon completion of the training procedure, an optimized model weights file was generated. The fine-tuned YOLOv11 model, combining the general knowledge encoded in the pretrained architecture with the task-specific information acquired during transfer learning, was subsequently implemented in the inference pipeline. This enabled fully automated, rapid detection and annotation of previously unseen test images (Fig. 5).

Statistical analyses

Statistical analyses were conducted in R Studio version 2026.01.1. Libraries used: performance, tidyverse, DHARMa, glmmTBR, lme4, lmerTest. To analyze eggshell microstructural characteristics, normality of the distribution was checked, the Shapiro-Wilk test or the Lilliefors test was performed, depending on the sample size. None of the data followed a normal distribution. Generalized Linear Mixed Models (GLMMs) were fitted using distributions based on the fit of each response variable. All models included egg laying order and season as fixed effects, while individual female ID was specified as a random effect to account for repeated measures and estimate the Intraclass Correlation Coefficient (ICC). Depending on the distribution of the eggshell structure data, the effect of female age on eggshell parameters was assessed using either the Kruskal–Wallis test followed by Dunn's post hoc test or one-way ANOVA followed by Tukey's post hoc test. All differences were considered statistically significant at $P < 0.05$.

Declarations

Competing interests

The author(s) declare no competing interests.

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Author Contribution

J.R: conceptualization, investigation, writing; R.G statistical analysis; S.N: preparing AI-based script, S.G: preparing samples, investigation,P.B: material collection, writing

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Data Availability

The datasets generated and/or analysed during the current study are available in the Repozytoria Uniwersytetu Przyrodniczego we Wrocławiu repository, https://bazawiedzy.upwr.edu.pl/info/researchdata/UPWRb57e699948d44436a169f99108f3f4b6?ps=20&tab=files&lang=en&title=Dane%2Bbadawcze%2B%25E2%2580%2593%2BDane%2Bbadawcze%2B%25E2%2580%2593%2BUniwersytet%2BPrzyrodnicz (DOI: 10.57755/80dr-rq19)

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Figures

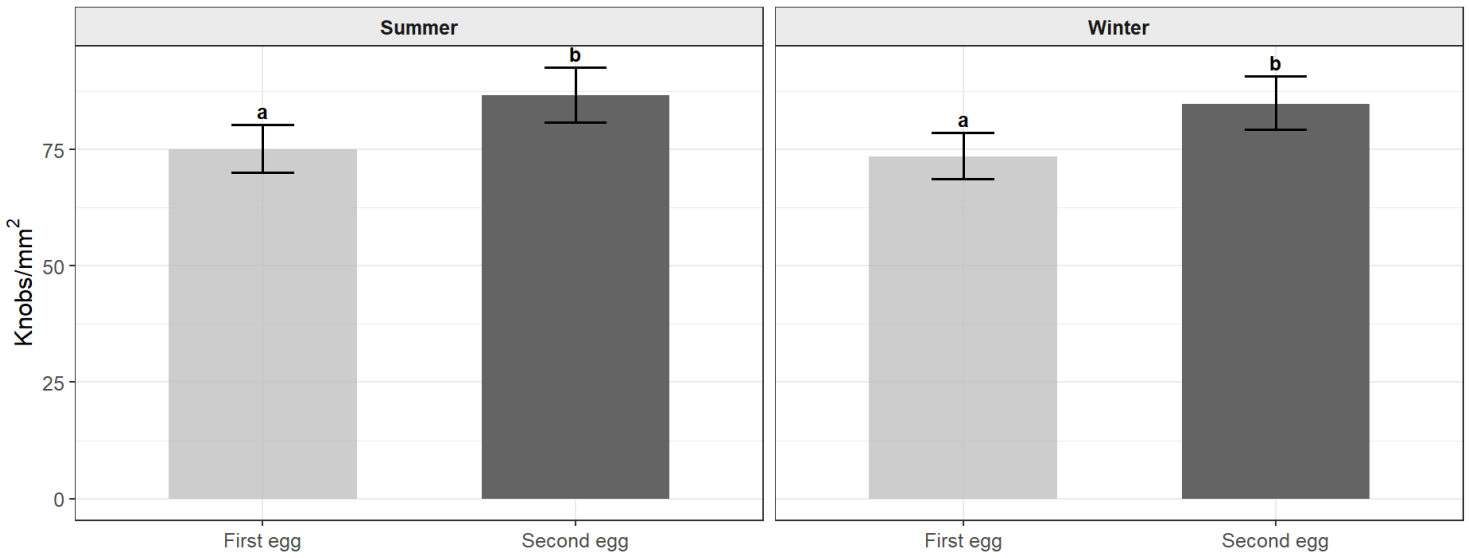


Figure 1

Differences in the number of mammillary knobs (per mm^2) between the first and second egg in the studied periods

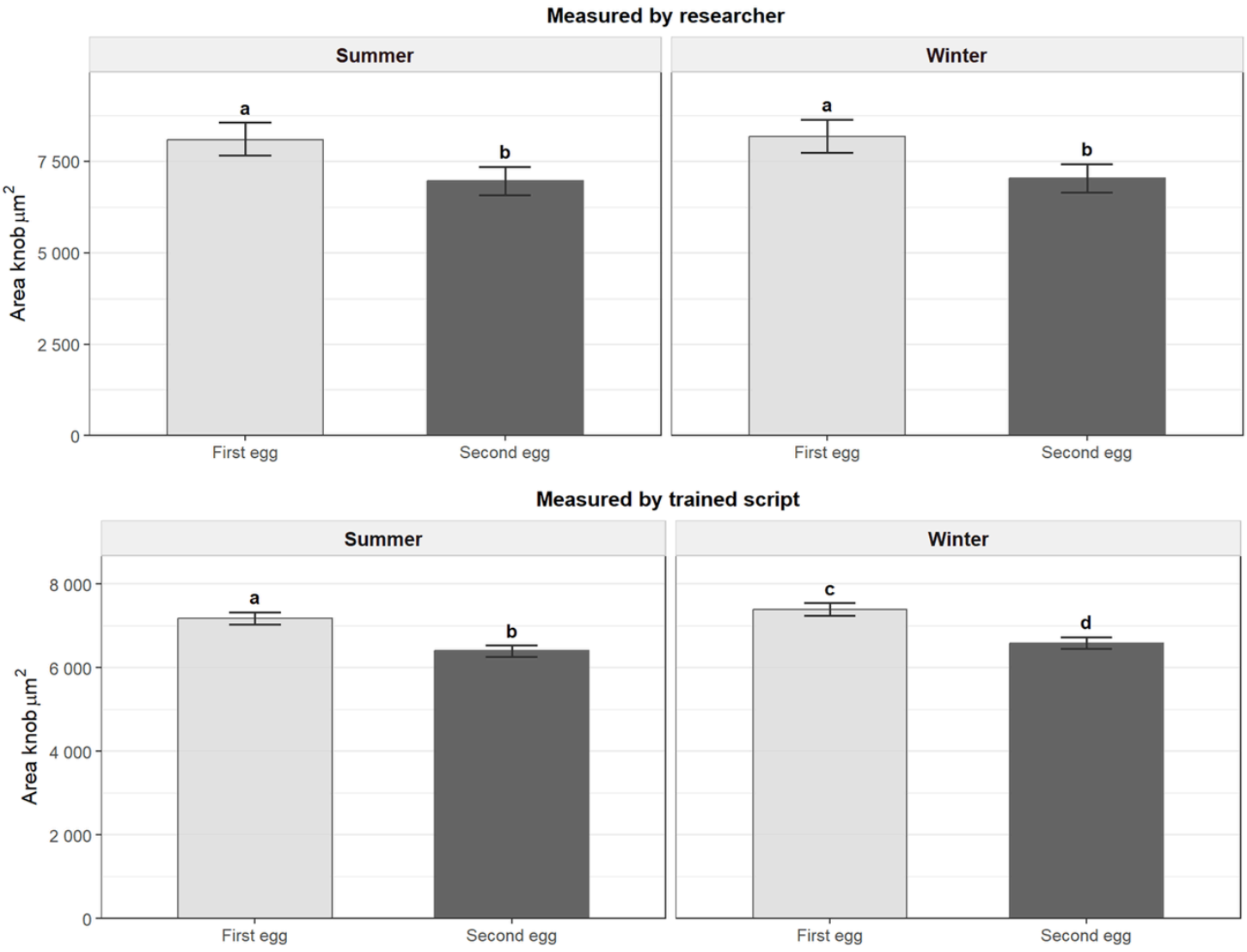


Figure 2

Differences in knob size between the first and second egg in the study periods (measurements made manually by the researcher and the trained AI-based script)

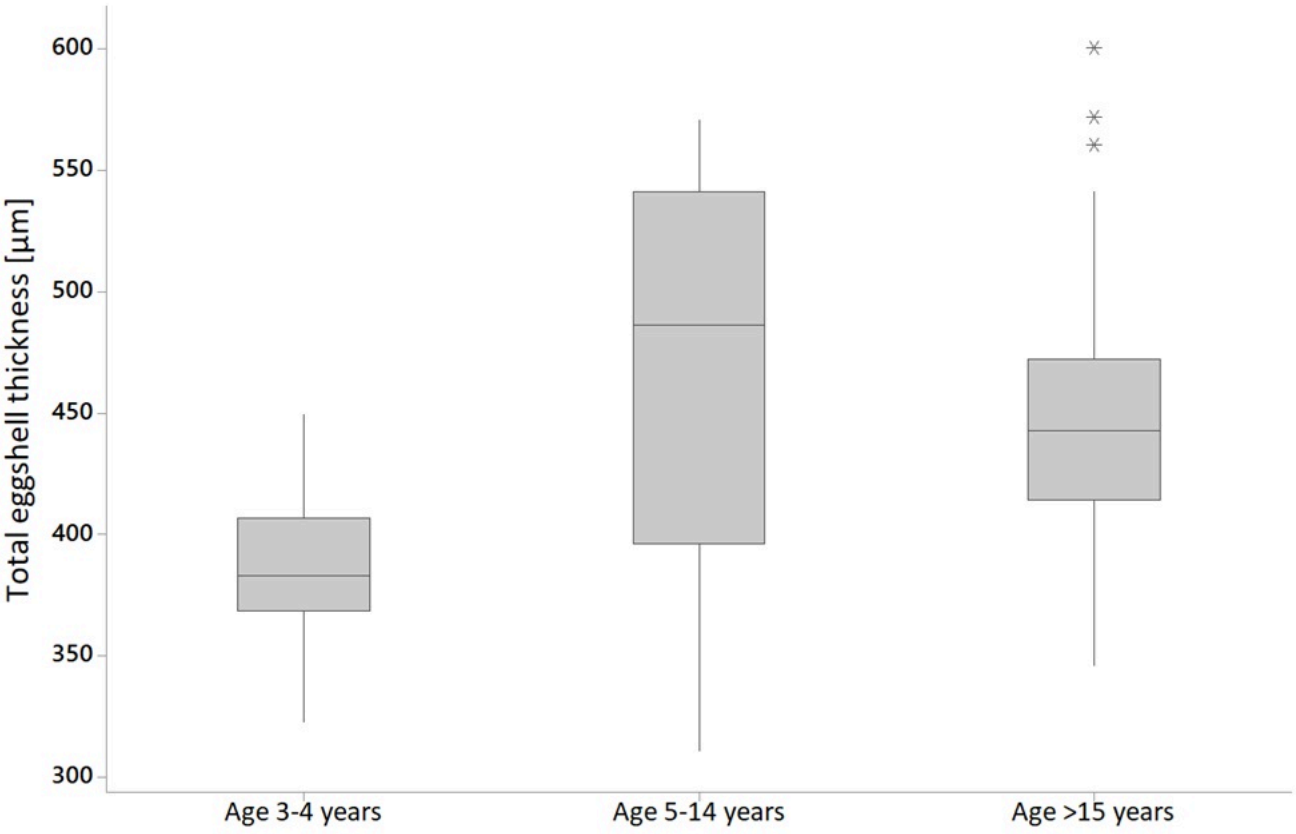


Figure 3

Boxplot of penguin eggshell thicknesses in different age categories

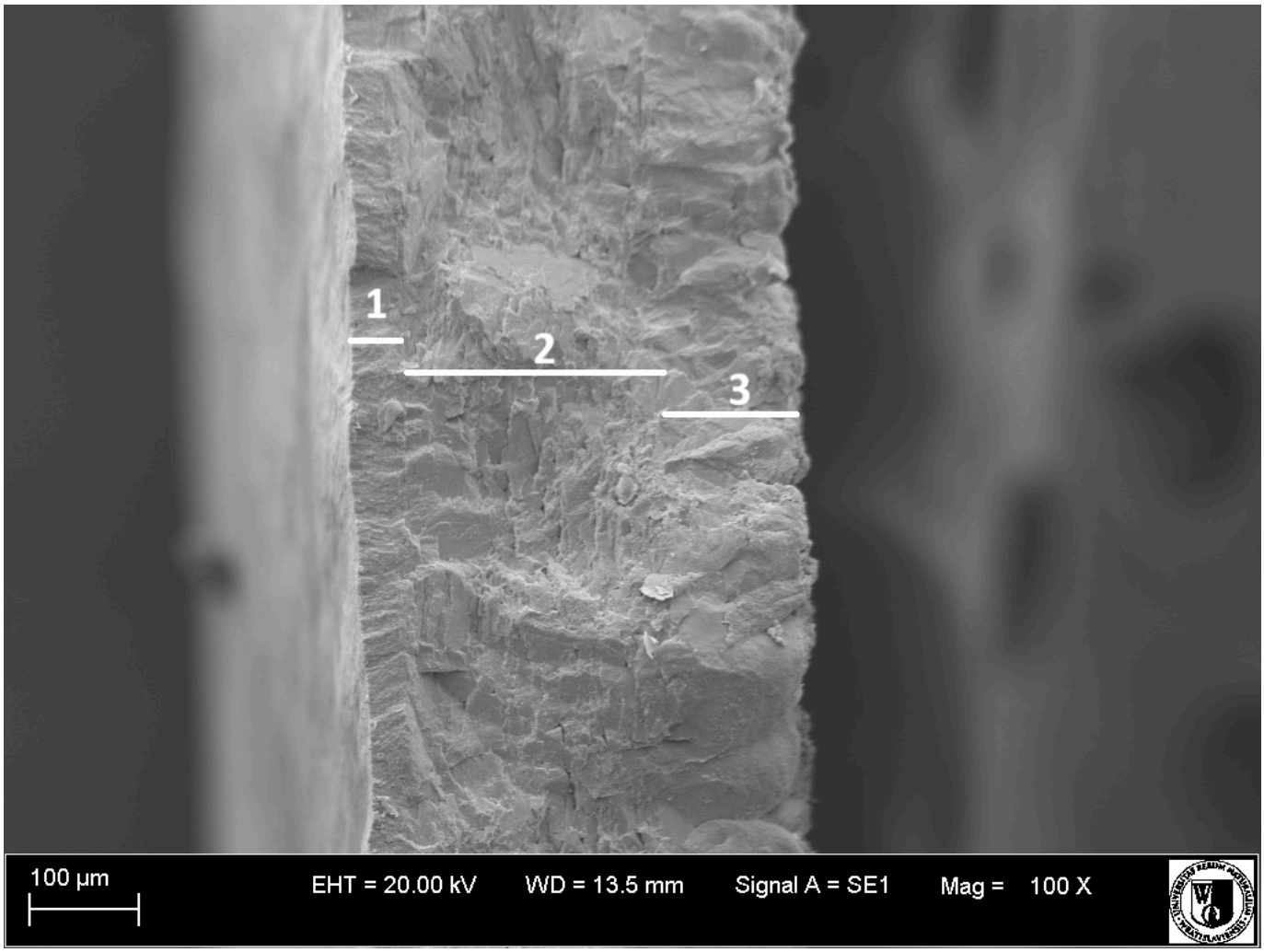


Figure 4

Marked layers of the African penguin's eggshell: 1 - crystalline layer; 2 - palisade layer; 3 - mammillary layer

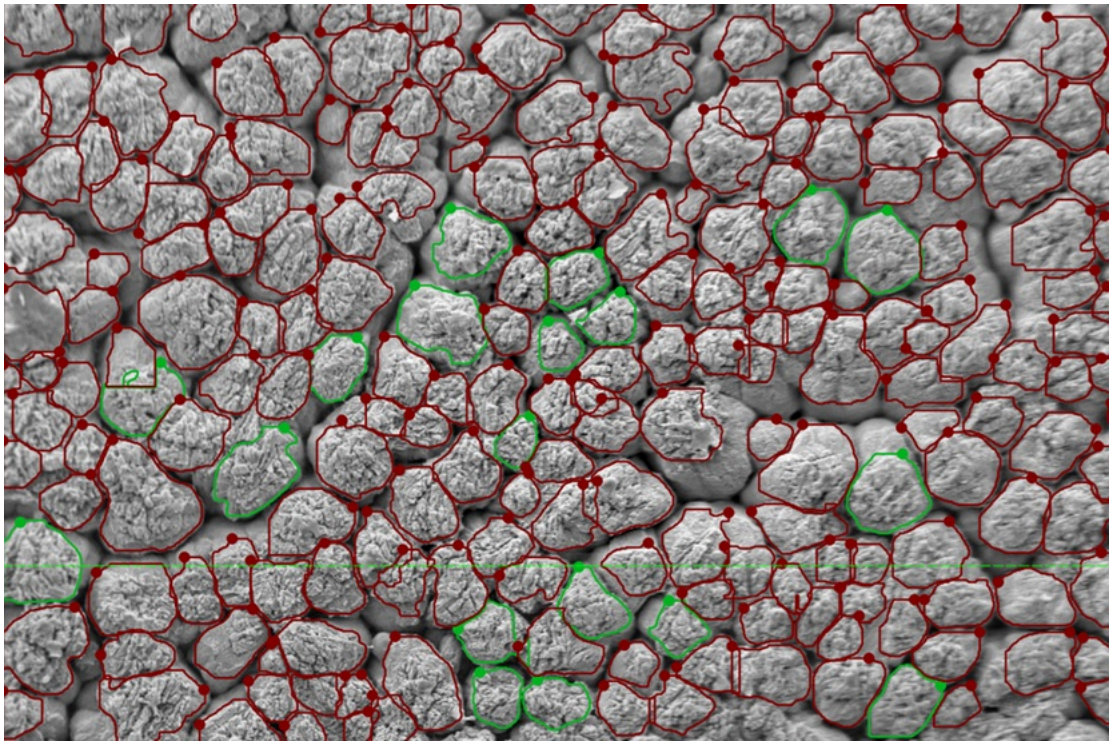


Figure 5

Marked by AI-based script knobs of the mammillary layer.

Supplementary Files

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