

Increase in membrane thickness during development compensates for eggshell thinning due to calcium uptake by the embryo in falcons

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Abstract We compared membrane thickness of fully developed eggs with those of non-developed eggs in different endangered falcon taxa. To our knowledge, membrane thickness variation during development has never been examined before in falcons or any other wild bird. Yet, the egg membrane constitutes an important protective barrier for the developing embryo. Because eggshell thinning is a general process that occurs during bird development, caused by calcium uptake by the embryo, eggs are expected to be less protected and vulnerable to breakage near the end of development. Thus, egg membranes could play an important protective role in the later stages of development by getting relatively thicker.

We used linear mixed models to explore the variation in membrane thickness ($n=378$ eggs) in relation to developmental stage, taxon, female age, mass and identity (73 females), egg-laying sequence (105 clutches) and the study zone. Our results are consistent with the prediction that egg membranes are thicker in fully developed eggs than in non-developed eggs, suggesting that the increase in membrane thickness during development may compensate for eggshell thinning. In addition, our data shown that thicker membranes are associated with larger, heavier and relatively wider eggs, as well as with eggs that had thinner eggshells. Egg-laying sequence, female age and the study zone did not explain the observed variation of membrane thickness in

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the falcon taxa studied. As we provide quantitative data on membrane thickness variation during development in falcons not subjected to contamination or food limitation (i.e. bred under captive conditions), our data may be used as a reference for studies on eggs from natural populations. Considering the large variation in membrane thickness and the multiple factors affecting on it and its importance in the protection of the embryo, we encourage other researchers to include measurements on membranes in studies exploring eggshell thickness variation.

Keywords Egg membrane · Eggshell thickness · Egg-laying sequence · Hybrid · Conservation · Raptor · CITES

Introduction

The avian egg provides the developing embryo with an ideal environment for development (Romanoff and Romanoff 1949; Rahn and Ar 1974; Solomon 1991). The eggshell membrane is a smooth layer of homogeneous dense material that functions as a site for respiration, nutrient transport, and waste storage during embryonic development (Kutchai and Steen 1971; Peakall et al. 1983; Cobb et al. 2003; Pepper et al. 2004). Membranes also prevent the embryo from drying out (Mao et al. 2007) and facilitate transport of calcium during late embryonic stages (Elaroussi and Deluca 1994; Ecay et al. 2004). Membranes also act as a filter against the penetration of microorganisms into the egg (Ahlborn and Sheldon 2005; Chao et al. 2007).

Membrane thickness has received very little attention in the literature (but see Yoshizaki and Saito 2002; Mao et al. 2007; Feberwee et al. 2009) when compared to eggshell thickness. Eggshell thinning has been the focus of much research in falcons, mainly because of the reproductive failure that have occurred in many parts of the northern hemisphere from about 1950 possibly due to persistent pollutants (Cade et al. 1971; Bunck et al. 1985; Bennett 1995; Peakall and Lincer 1996; Wegner et al. 2005; Falk et al. 2006; Kamata et al. 2006; Jagannath et al. 2008; Castilla et al. 2009a). Embryo development, however, also causes eggshell thinning in many bird species (Vanderstoep and Richards 1970; Bunck et al. 1985; Castilla et al. 2007; Karlsson and Clas Lilja 2008; Fernie et al. 2009) including falcons of different taxa (Castilla et al. 2010), as the embryos utilise minerals and calcium from the eggshell to assist in the development of the skeleton, muscles and brain (Tuan et al. 1991; Blom and Lilja 2004; Wilkin et al. 2009). One means to compensate for eggshell thinning in some birds is the deposition of a thicker layer of shell pigment (Gosler et al. 2005). Another way to compensate for eggshell thinning that would provide protection for the

embryo during the latest phase of their development would be to increase the thickness of the egg membranes.

In this study, we examined the effect of developmental stage on membrane thickness variation in different falcon taxa. We tested the prediction that membranes from fully developed eggs are thicker than those from non-developed eggs. We additionally explored the effect of different egg characteristics, female traits, egg-laying sequence and study zone on membrane thickness variation.

Materials and methods

Study animals and eggs

Falcons are included in the list of the Convention of International Trade of Endangered Species (CITES; Tucker and Heath 1994). In addition, gaining access to their nests and eggs is very difficult because they are normally in sharp cliffs. Consequently, there is a large gap in our knowledge regarding membrane or eggshell thickness variation and the factors affecting on it.

The falcon species examined in this study are protected, rare or endangered and include the peregrine falcon (*Falco peregrinus peregrinus*; P), the red shaheen falcon (*Falco peregrinus babylonicus*; R); the intraspecific hybrid peregrine×red shaheen (*F. peregrinus peregrinus*×*F. peregrinus babylonicus*; PR); the saker falcon (*Falco cherrug*; S), the gyr falcon (*Falco rusticolus*), and the interspecific hybrid between the saker falcon (*F. cherrug*) and the gyr falcon (*F. rusticolus*; SG). Females show large differences in body size (ranging from 600 to 1,800 g) depending on the taxon (Cramp and Simmons 1980). In this study, body mass was measured at the end of summer after reproduction was finished. The birds were captured in their cages and weighed with an electronic balance to the nearest gramme (KRUPS 840, Germany). The age of the females was provided by the one responsible for the breeding centre “Roc Falcon” who has information on the birth date of all individuals.

The eggs were obtained from two different breeding zones in Catalonia (NE Spain) that were not subjected to agricultural or industrial pollution. One zone was near the coast (GI) at 97 m.a.s.l. (mean annual temperature=14°C, mean annual precipitation=650 mm, mean relative humidity=80%); the other was in the Pyrenees mountains (RF) at 800 m.a.s.l. (mean annual temperature=12°C, mean annual precipitation=650 mm, mean relative humidity=65%).

Falcons are bred worldwide and a common target for conservation programmes (Rahbek 1993). This provides a unique opportunity where a large sample of eggs can be obtained for scientific research. Moreover, falcons in captive breeding facilities are bred under optimal conditions

and fed high quality food. Since all taxa and individuals are treated similarly, results can be compared without bias in that respect. We examined 105 clutches from 73 different females (total of 211 non-developed and 167 developed eggs) that were collected between March and July of 2007.

In the field, the falcon species studied here typically have one or two clutches from four to six eggs and an incubation period that last ca. 25 days (Cramp and Simmons 1980). Egg pulling (i.e. removing eggs as they are laid) was conducted in both zones, so females did not produce true clutches as they would do in the wild. However, most females produced between four and nine eggs. Data on egg-laying sequence was obtained by writing down a number on the eggshell as the female was laying it. The eggshells from hatched chicks were obtained from the birth rooms.

We used an ovoscope and direct observations after egg breakage to establish two developmental categories: eggs with non-developed embryos (i.e. infertile or aborted during the first week of incubation; Fig. 1), and developed eggs (i.e. hatched eggs or aborted eggs with fully developed embryos). Egg size (length and width) was measured with a calliper (Mitutoyo) to the nearest 0.01 mm. Egg mass was measured in fresh eggs that were recently laid with an electronic Sartorius AG, balance Goettingen, Germany (to 0.01 g). Membranes were separated from the eggshells after immersing them in a plastic box with water for 10 min. In

eggs of both developmental stages, we only measured the outer thick eggshell membrane after cleaning it with water and removing the thin vascularized chorioallantoic membrane (Fig. 1). Membrane thickness was measured using a micrometer (Mitutoyo) to the nearest 0.001 mm. Measurements were taken in a similar way, that is, by compressing the membrane with a standardised pressure provided by the spring in the micrometer. Membrane and eggshell thickness were measured in one site around the equator of the egg. All measurements were done by the same person; we are confident that these are perfectly comparable among species.

Statistical procedures

Because all egg dimensions (length, width, mass and shell thickness) as well as female mass were highly correlated ($r > 0.4$ and $p < 0.0001$ in all cases), we based all analyses on a multivariate summary of the these data. We first performed a principal component analysis to reduce the dimensionality of the data set. This resulted in a new set of uncorrelated variables that can be analysed separately. Results of the principal component analysis were represented graphically using biplots, where the cosine of the angle between two vectors provides an estimate of the correlation between the respective variables. Principal components were used as independent variables in a mixed

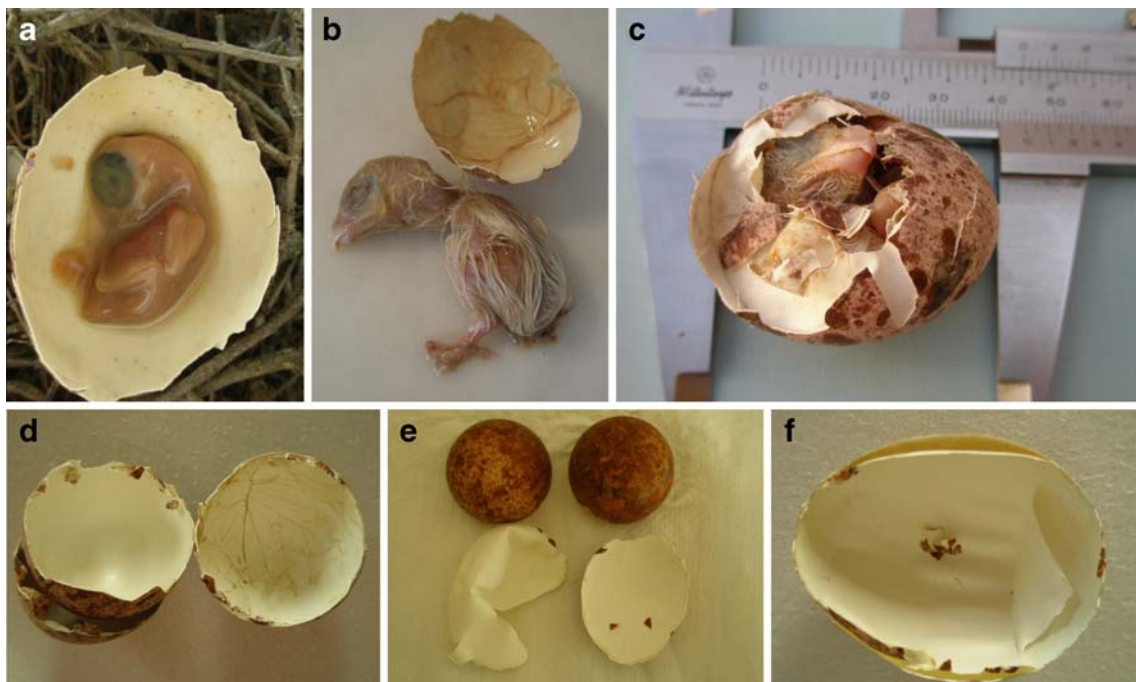


Fig. 1 Eggshells, membranes and embryos of different developmental stages in falcon species. **a, d, e** Peregrine falcon (*Falco peregrinus peregrinus*). **b** Intraspecific hybrid peregrine × red shaheen (*F. peregrinus peregrinus* × *F. peregrinus babylonicus*). **c** Red shaheen falcon (*Falco peregrinus babylonicus*). **f** Interspecific

hybrid saker × gyrfalcon (*Falco cherrug* × *Falco rusticolus*). The chorioallantoic membrane (vascularized) is present in nearly fully developed embryos (**b, c**) and in hatched eggs (**d, right**). Membranes are also shown in infertile eggs (**d left, e, f**)

linear model with membrane thickness as dependent variable. Additionally, developmental stage and zone were added as fixed factors. Laying sequence and female age were added as continuous covariates. Female identity was added as a random effect to estimate among-female variation and to take the dependency of the data for eggs from a single female into account. Tests of fixed effects were based on F tests with degrees of freedom approximated by Kenward and Rogers method. The random female effect was tested using a likelihood ratio test. The data was analysed using linear mixed models in SAS (version 9.2).

In this study, we have compared individuals of different species that cannot be treated as statistically independent data points following the premise put forth by Joe Felsenstein (1985, 2008). However, the number of falcon species that we studied here is too low ($n=3$), thus providing very few degrees of freedom if one were to do an independent contrast analysis on the species means. Moreover, some of our data points are hybrids, making it conceptually hard to include them as if they were ‘independent’ species. We have to be aware of this limitation in the present study.

Results

The smaller falcons of the peregrine group (P, R, PR) have egg membranes significantly thinner than the big falcons (S, G, SG) when considering both developmental stages

separately (developed: analysis of variance (ANOVA), $F_{1,208}=26.76$, $p<0.001$; undeveloped: ANOVA, $F_{1,166}=9.16$, $p=0.003$; Table 1, Fig. 2). We also found a high significant correlation between membrane thickness and eggshell thickness in eggs under different developmental stages: undeveloped eggs (regression on Log_{10} -transformed data: $r=0.37$; $p<0.001$; slope=0.94; intercept=-0.71) and fully developed eggs (regression on Log_{10} -transformed data: $r=0.32$; $P<0.001$; slope=0.60; intercept=-0.79; Fig. 2).

Because several of the morphometric measurements were highly correlated, we conducted first a principal component analysis, and found that the first three components explained 93% of all variation (Table 2). Eigenvectors and a graphical representation of this principal component analysis as biplots are given in Table 2 and Fig. 3. The first principal component corresponded to an overall size measure (egg and female size). The second principal component was determined by a contrast between egg length and shell thickness. Higher scores for the second component correspond to relative long eggs with relative thin shell. Finally, the third principal component was determined by a contrast between egg length and shell thickness on the one hand and egg width and female mass on the other hand. The strong correlations showed by the principal component analysis allow imputing the missing data by using multiple imputations. After that, all data can be used to see which factors are correlated with membrane thickness (Table 3).

Table 1 Measurements of membrane thickness (in millimetres) for eggs with fully developed embryos or with non-developed embryos in different falcon taxa

Taxa	Zone	Developed eggs					Non-developed eggs				
		Mean	SD	Max	Min	N	Mean	SD	Max	Min	N
P	GI	0.088	0.006	0.094	0.077	9	0.066	0.015	0.1	0.037	32
	RF	0.077	0.016	0.101	0.036	29	0.059	0.019	0.095	0.019	40
R	GI	0.071	0.015	0.092	0.035	14	0.054	0.018	0.09	0.025	28
	RF	0.061	0.018	0.081	0.026	7	0.051	0.020	0.087	0.02	16
PR	GI	0.066	0.019	0.105	0.038	10	0.059	0.009	0.065	0.044	6
	RF	–	–	–	–						
S	GI	0.070	0.011	0.093	0.054	9	0.069	0.014	0.098	0.048	15
	RF	0.083	0.014	0.102	0.053	30	0.074	0.012	0.094	0.057	20
G	GI	–	–	–	–						
	RF	–	–	–	–		0.076	0.007	0.088	0.066	9
SG	GI	0.072	0.013	0.091	0.054	6	0.081	0.007	0.091	0.073	7
	RF	0.084	0.013	0.106	0.054	53	0.068	0.020	0.108	0.032	38

Indicated are the means and standard deviations (SD), the maximum and minimum values, the sample size (N) and the zone (see “Materials and methods”)

P peregrine falcon (*Falco peregrinus peregrinus*), R=red shaheen falcon (*Falco peregrinus babylonicus*), PR intraspecific hybrid peregrine×red shaheen (*F. peregrinus peregrinus*×*F. peregrinus babylonicus*), S saker falcon (*Falco cherrug*), G gyr falcon (*Falco rusticolus*), SG interspecific hybrid saker×gyr (*F. cherrug*×*F. rusticolus*)

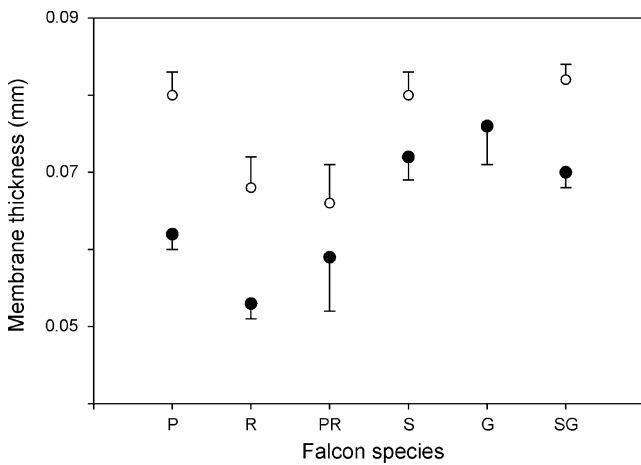


Fig. 2 Membrane thickness (mean±SE) of eggs of different developmental stage (*white*=undeveloped; *black*=fully developed) for the different falcons. *P* peregrine falcon (*Falco peregrinus peregrinus*), *R* red shaheen falcon (*Falco peregrinus babylonicus*), *PR* intraspecific hybrid peregrine×red shaheen (*F. peregrinus peregrinus*×*F. peregrinus babylonicus*), *S* saker falcon (*Falco cherrug*), *G* gyr falcon (*Falco rusticolus*), *SG* interspecific hybrid saker×gyr (*F. cherrug*×*F. rusticolus*)

Membranes were significantly thicker in eggs at a later developmental stage where fully developed eggs had thicker membranes than non-developed eggs (Fig. 2, Table 3). For all falcon taxa, membrane thickness was also correlated with egg size and female body mass (i.e. a positive correlation with the first principal component, Figs. 3 and 4), and this correlation did not differ between the two developmental stages (i.e. no two-way interaction, Table 3). Thicker membranes corresponded to relatively long eggs with thinner eggshells (i.e. correlation with PC2, Table 3, Fig. 4). Species, zone, egg-laying sequence and the third principal component did not explain the observed membrane thickness variation in falcon taxa (Table 3, Fig. 5).

Discussion

In this study, we provide the first quantitative data on membrane thickness variation during development for non-

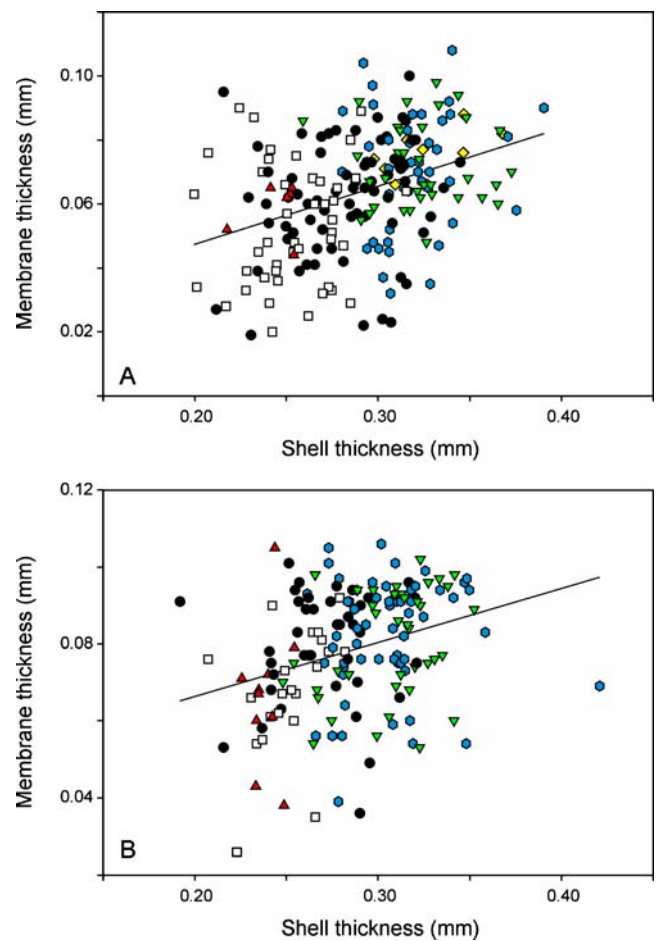


Fig. 3 Relationship between eggshell thickness and membrane thickness for undeveloped eggs (**a**) and for fully developed eggs (**b**). *Black circle*: peregrine falcon, *P* (*Falco peregrinus peregrinus*); *white square*: red shaheen falcon, *R* (*Falco peregrinus babylonicus*); *red triangle up*: intraspecific hybrid peregrine×red shaheen, *PR* (*F. peregrinus peregrinus*×*F. peregrinus babylonicus*); *green triangle down*: saker falcon, *S* (*Falco cherrug*); *yellow diamond*: gyr falcon, *G* (*Falco rusticolus*), *blue hexagon*: interspecific hybrid saker×gyr, *SG* (*F. cherrug*×*F. rusticolus*)

poultry birds. However, as the falcons in our study were raised in optimal conditions, this allows our data to be used as a reference for studies on eggs from natural populations. Our results are consistent with the prediction that membrane thickness increases during incubation and suggest an

Table 2 Eigenvectors and proportion variance explained by the principal component analysis of egg characteristics and female mass (a screen plot is given in Fig. 3)

	PC1	PC2	PC3	PC4	PC5
Length	0.400928	0.764996	0.337077	0.104549	0.359843
Width	0.463732	-0.155637	-0.390096	-0.682461	0.377892
Egg mass	0.507505	0.175252	-0.077946	-0.111582	-0.832586
Shell thickness	0.404397	-0.559875	0.720487	0.022831	0.058141
Female mass	0.450679	-0.215372	-0.457195	0.714384	0.17644
% Variance explained	70	12	9	6	3
Cumulative %	70	82	91	97	100

Table 3 Overview of significance tests of associations between membrane thickness in different falcon taxa and explanatory variables

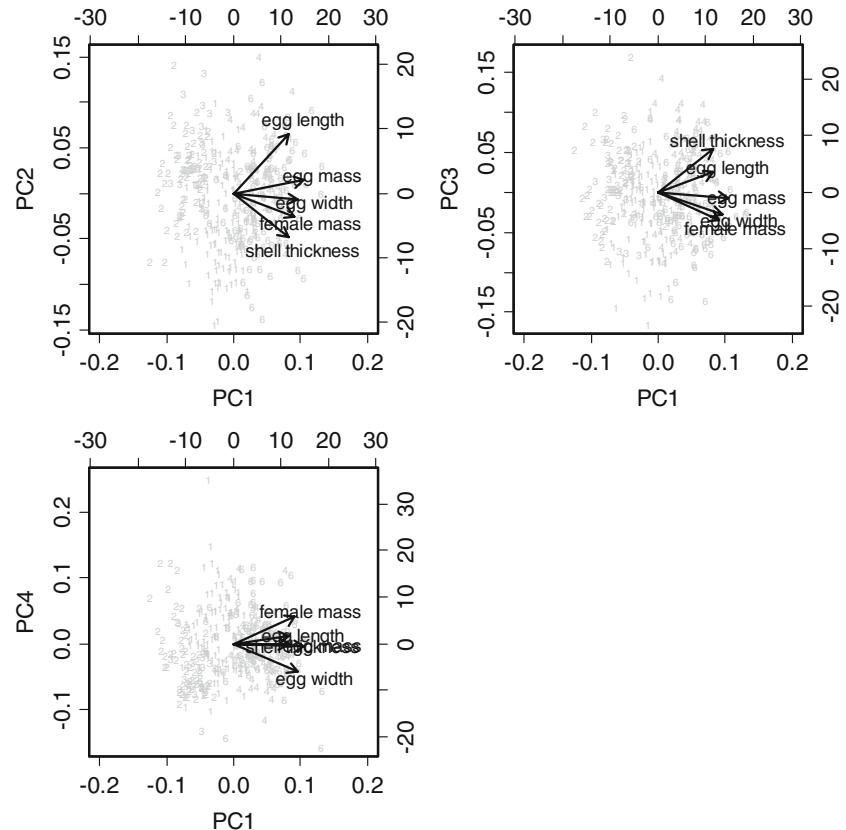
Parameter	F value	df 1	df 2	p value
PC1	30.0	1	89	<0.0001
PC2	10.4	1	302	0.001
PC3	1.99	1	354	0.16
Developmental stage	67.8	1	365	<0.0001
Egg-laying sequence	0.01	1	53	0.90
Female age	0.44	1	59	0.51
Zone	1.70	1	54	0.20
PC1×developmental stage	1.57	1	349	0.21
PC2×developmental stage	0.05	1	353	0.82
PC3×developmental stage	0.09	1	336	0.76

important effect on the protection of the developing embryo. This should be especially relevant for bird species nesting in rocky areas, such as falcons (Cramp and Simmons 1980). The egg membrane can act to prevent global cracking of the eggs in case of small cracks, as it provides a strong coherence among the parts of the eggshell. A small crack in the eggshell need not be fatal to the embryo. As long as the egg membrane is intact, the embryo is shielded from external influences such as pathogens and some fluctuation in environmental conditions.

A similar trend towards an increase in membrane thickness during development was observed in the red-legged partridge (*Alectoris rufa*; Castilla and Herrel, unpublished). Our results are also in agreement with the findings of a recent study (Feberwee et al. 2009) which revealed that when the mammillary layer of the calcified zone of the egg was absent, the inner eggshell membranes were thicker.

In contrast to our observations, shell membranes in farm quails become thinner during incubation (Yoshizaki and Saito 2002), which has been suggested to be related to the need to satisfy the requirements for gas exchange (Kutchai and Steen 1971). However, to make comparisons across studies and species, similar methods should be employed since the structure of egg membranes (as demonstrated by electron micrographs) is different in the region of the air chamber compared to other regions of the egg (Mao et al. 2007). In our study, we used the same micrometer, and we measured membrane thickness always in the same site around the equator in all eggs. Also, the measurements of developed and non-developed eggs were conducted during the same time. Thus, our results showing the same trend of membrane thickness increase during development in all the five falcon taxa examined are not likely the result of a measurement artefact.

Fig. 4 Biplots of the three first principal components of a principal component analysis of egg measurements and female mass. Data from the different species are indicated by different numbers: 1=peregrine falcon (*Falco peregrinus peregrinus*); 2=red shaheen falcon (*Falco peregrinus babylonicus*); 3= intraspecific hybrid peregrine× red shaheen (*F. peregrinus peregrinus*×*F. peregrinus babylonicus*); 4=saker falcon (*Falco cherrug*); 5=gyr falcon (*Falco rusticolus*); 6= interspecific hybrid saker×gyr (*F. cherrug*×*F. rusticolus*)



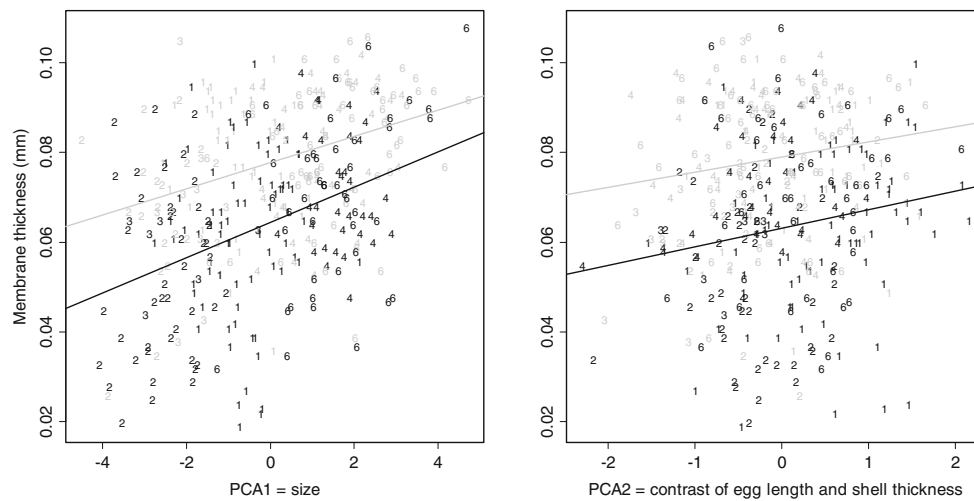


Fig. 5 Associations between membrane thickness and the first and second principal component of egg measurements and female mass for fully developed (grey) and non-developed (black) eggs in different falcon taxa. Different species are indicated by different numbers: 1=peregrine falcon (*Falco peregrinus peregrinus*); 2=red

shaheen falcon (*Falco peregrinus babylonicus*); 3=intraspetic hybrid peregrine×red shaheen (*F. peregrinus peregrinus*×*F. peregrinus babylonicus*); 4=saker falcon (*Falco cherrug*); 5=gyr falcon (*Falco rusticolus*); 6=interspecific hybrid saker×gyr (*F. cherrug*×*F. rusticolus*)

In contrast to our expectations, egg-laying sequence did not have an effect on membrane thickness. Yet, a decrease in mean shell thickness in later clutches of captive peregrine falcons (Burnham et al. 1984; Falk and Møller 1990) and in other falcon taxa (Castilla et al. 2010) has been observed. An additional factor that could influence membrane thickness variation with respect to egg-laying sequence is variation in chick mass related to sex (females are much bigger in falcons). However, since information on the sex of the developing embryos was not available, this needs to be investigated further.

We found that heavier females produced larger and heavier eggs with thicker membranes. Our results are in agreement with other authors who hypothesised that for an effective incubation without breaking the eggs in the nests, big birds have to produce bigger and thicker eggs than small birds (Ar et al. 1979). We also found that the age of the female did not influence on membrane thickness variation in this study. This result was rather surprising since a previous study on falcons (albeit using infertile non-developed eggs) found that older females produced eggs with thinner membranes than younger females (Castilla et al. 2009b). Because we used different females of different age and condition in two studies, this may have influenced the different trend in our results with younger females being included in the present study. However, this variation is biologically relevant and important and should be taken into consideration. Additional data on how female age influences egg and egg membrane characteristics are needed to gain a fuller understanding of the observed patterns.

In our study, membrane thickness variation among taxa was not significantly different between falcons from

different zones, suggesting that falcons in both areas were subjected to overall similar conditions. Moreover, these results indicate that the differences in climate and altitude across the study zones did not affect on membrane thickness.

Given the large variation in membrane thickness and its potential importance in the protection of the embryo, we encourage other researchers to include measurements on egg membranes in studies exploring eggshell thickness variation. We also suggest that researchers and field naturalists should be able to determine whether eggs are infertile, aborted or hatched based on collections of pieces of eggshells from falcon nests, which could avoid erroneous interpretations of nest failure due to factors not related to embryo development. We argue that an increased attention paid to membranes would be beneficial and suggest that field researchers should collect and store eggshells for further research when possible, since they are non-invasive indicators of environmental or microbial contamination, the nutrient source of females during egg formation, a source of DNA for genetic studies and an excellent source for the detection of viral infections (Pearce et al. 1997; Miller et al. 2003; Chao et al. 2007; Opperl et al. 2009).

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