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Comparison of micrometer- and scanning electron microscope-based measurements of avian eggshell thickness

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ABSTRACT. The study of avian eggshell structure, including composition, pigmentation, thickness, and strength, has important ecological and economic implications. Previous investigators have used a variety of techniques to derive either direct measures or indirect estimates of eggshell thickness. Assessing the repeatability and method agreement of different techniques is necessary to permit comparison of eggshell thickness values from different studies on various genetic stocks, populations, and species. We recorded and analyzed measurements of eggshell thickness using two methods, micrometers and scanning electron microscopy (SEM), for several Palaeognathae and Neognathae taxa, including nonpasserines and passerines. Applying a tolerance-interval approach, we found that repeatability of measurements for eggs with thinner shells (<300 μm, all Neognathae taxa) was worse than for eggs with thicker shells (Palaeognathae taxa), but was still statistically and biologically reasonable given that the relative magnitude of intramethod agreements was <11%. Our results support previous predictions that measurements made using a micrometer are comparable to those made using SEM. This finding is particularly important given the relative ease and cost efficiency of the micrometer method. Importantly, these new analyses can be used to validate the use of published data from previous studies of micrometer-based eggshell thickness for both intra- and interspecific comparisons.

RESUMEN. **Comparación entre un micrómetro y un microscopio electrónico (MEB) en la medida del grosor de cascarones de huevos de aves**

El estudio de la estructura del cascarón de los huevos de aves, incluyendo su composición, pigmentación, grosor y fortaleza, tiene implicaciones ecológicas y económicas de importancia. Los trabajos que se han publicado sobre el grosor del cascarón utilizan una amplia variedad de técnicas para determinar directamente el grosor o hacer estimados indirectos. La forma de poder repetir las medidas y el método a seleccionarse, entre diferentes técnicas, es necesario para poder comparar el grosor del cascarón en diferentes estudios ya sea de diferentes grupos genéticos, poblaciones o especies. Nosotros tomamos y analizamos las medidas del grosor de cascarones de huevos utilizando dos métodos: el uso del micrómetro y un microscopio electrónico de barrido (MEB). Estos se utilizaron tanto en Palaeognathae y Neognathae, incluyendo paserinos, como no-paserinos. Aplicando un enfoque de intérvalo de tolerancia, encontramos que el repetir una medida para huevos con cascarón fino (<300 μm, todos de Neognathae), era peor que para huevo con cascarón más gruesos (Palaeognathae), aunque biológico y estadísticamente razonables, dado el caso de que la magnitud relativa de armonía entre métodos, fue <11%. Nuestros resultados apoyan predicciones previas de que las medidas tomadas usando un micrómetro son comparables a aquellas tomadas con un MEB. Esto es particularmente importante por que el método del micrómetro es fácil y costo efectivo. Más importante aún es que este estudio comparativo permite el validar los datos publicados en

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estudios previos utilizando micrómetros para determinar el grosor de los huevos, tanto en estudios intra- como inter-específicos.

Key words: avian eggshell, electron microscope, thickness measurement, tolerance interval

The study of eggshell structure is important for economic (Gonzalez et al. 1999), evolutionary-ecological (Picman and Pribil 1997), and ecotoxicological (Albanis et al. 1996, Pain et al. 1999) reasons. A number of shell characteristics, including thickness and pigmentation, have direct or putative links to eggshell strength (Silyn-Roberts and Sharp 1986, Picman 1989, Gosler et al. 2005). Eggshell thickness varies, being thinnest and most uniform at the equator and thickest at the poles. The equatorial zone is also the largest portion of the egg surface area relative to the surface areas represented by the other regions and, therefore, can provide the best estimate of the lower limit of overall strength; thickness is most frequently measured in this region (Voisey and Hunt 1974).

Methods for directly measuring eggshell thickness (Hoyt 1979) have included the use of microscopes, mechanical gauges (calipers, dial gauges, and micrometers), and ultrasound (Rothstein 1972, Spaw and Rohwer 1987, Moksnes et al. 1991), whereas indirect estimates of thickness are based on formulae involving eggshell size and shell weight (Schönwetter 1960–1992, Ratcliffe 1967, Ar et al. 1979, Rahn and Paganelli 1989, Maurer et al., in press). Mechanical equipment may sometimes be unsuitable due to the fragility of small eggs, especially in museum collections, and so a calculated index of thickness is often the only feasible, or permissible, approach (Szaro et al. 1979, Pain et al. 1999). The accuracy and comparability of different mathematical equations for eggshell thickness are described in detail by Green (2000). Studies assessing direct data of micrometer-based measures of thickness and mathematically derived indirect indices of thickness have also revealed strong correlations between these two techniques (Heinz 1980, Green 1998). Thus, using a combination of methods enables comparative analyses of thickness (e.g., Mermoz and Ornelas 2004).

No study to date, however, has compared the two techniques most often used to directly measure eggshell thickness. Quantitative comparisons across a range of scales are important because both the magnitude of variability and the absolute scale of measurements likely vary between eggshells of similar origins and dimensions (e.g., intraspecific samples) and those with different origins and dimensions (e.g., interspecific samples). Our objective was to compare measurements of eggshell thickness as measured using scanning electron microscopy (SEM) (Blankespoor et al. 1982, Booth and Seymour 1987) and a micrometer. Although no one to date has compared microscope and mechanical methods for measuring eggshell thickness, Booth and Seymour (1987) suggested that SEM-based thickness estimates would be similar to micrometer-based data because both techniques involve direct measurements at specific points of eggshells.

METHODS

Eggshell samples. We measured a broad taxonomic sample of eggs from 15 species, including eight nonpasserines and seven passerines (Table 1). To estimate intraspecific agreement of the two measurement techniques, we used two samples of shells of: (1) hatched eggs of North Island Brown Kiwis (*Apteryx mantelli*; $N = 16$) and (2) unincubated eggs of different gentes of European Common Cuckoos (*Cuculus canorus*; $N = 7$). To estimate interspecific agreement of the two measurement techniques, we used shells of unincubated eggs of different Palaeognathae and Neognathae species. We acquired eggshells from a range of sources (Cassey et al. 2006), with two different eggs from every Neognathae species. Each eggshell fragment was from a different individual or nest.

Eggshells were cleaned with 70% ethanol. Inner membranes were removed to leave only the calcitic layers for measurement, and shells were placed in dark dry storage at −20°C until measurement. Cold storage was used to minimize any chemical changes in eggshell pigments (Igic et al. 2010).

Thickness measurements. All measurements of the same egg samples were taken by the same person (JAG or BI). Fragments

Table 1. Sample species names for each of the four data sets, including two intraspecific sample sets and two comparative sample sets, in the thickness method comparison analyses. The number of shells we measured is provided for all species. Eggs were collected fresh and unincubated, unless otherwise noted (* = incubated/hatched, ^ = unknown). Locations (or field sites) where the samples were collected under license are cited in the specific references.

Species	*N*	Location (reference)
Intraspecific sample sets		
Kiwi		
Apteryx mantelli	16	Operation Nest Egg, Rainbow Springs, Rotorua, New Zealand* (Igic et al. 2010)
Cuckoo		
Cuculus canorus	2	Finland (Grim et al. 2009)
Cuculus canorus	2	Hungary (Moskát and Hauber 2007)
Cuculus canorus	1	Japan (Lotem et al. 1995)
Cuculus canorus	2	Czech Republic (Grim 2007)
Comparative sample sets		
Palaeognathae		
Apteryx mantelli	2	Operation Nest Egg, Rainbow Springs, Rotorua, New Zealand* (Igic et al. 2010)
Family Dinornithidae (Moa)	1	Provided by Otago Museum, New Zealand^ (Igic et al. 2010)
Dromaius novaehollandiae	2	Northland Ostrich and Emu Ltd., Kaitaia, New Zealand (Igic et al. 2010)
Rhea americana	1	Commercial store in Berkeley, California, USA^ (Igic et al. 2010)
Struthio camelus	1	Northland Ostrich and Emu Ltd., Kaitaia, New Zealand (Igic et al. 2010)
Tinamus major	1	Estacion Biologica La Selva, Costa Rica (Igic et al. 2010)
Neognathae		
Coccyzus americanus	2	Union County, PA, USA (Dearborn et al. 2009)
Cuculus canorus	2	Hungary (Moskát and Hauber 2007); Czech Republic (Grim 2007)
Acrocephalus scirpaceus	2	Czech Republic (Grim 2007)
Acrocephalus arundinaceus	2	Hungary (Moskát and Hauber 2007)
Dumetella carolinensis	2	Union County, PA, USA (Dearborn et al. 2009)
Parus major	2	Finland (Grim et al. 2009)
Ficedula hypoleuca	2	Finland (Grim et al. 2009)
Phoenicurus phoenicurus	2	Finland (Grim et al. 2009)
Turdus philomelos	2	Benneydale, New Zealand (Cassey et al. 2009)

from the equatorial region of eggshells (Voisey and Hunt 1974) were removed and thickness measured at three randomly selected points using a commercial point micrometer (Series 112; Mitutoyo Corp., Kawasaki, Japan) (0–25 mm range, 0.01 mm graduation) and estimated to the nearest 0.001 mm. Previously measured fragments were then mounted on a metal stub, and a thin layer of platinum applied (using a Polaron SC 7640 SPUTTER COATER at 5–10 mA 1.1 kV for 3 min) to allow visualization of the eggshell cross-section under an SEM. A micrometer cannot be used at the cross-section edge of a shell fragment and an SEM cannot measure thickness outside of the cross-section edge. Thus, the SEM and micrometer measure subtly different aspects of eggshell thickness of the same fragment.

Digital photographs of eggshell cross-sections were taken at a magnification of 1000× using a Philips XL30S FEG SEM camera. Measurements of eggshell thickness on the photographs were taken using the ruler functionality on ImageJ 1.40g (National Institute of Health, USA; downloadable free from http://rsb.info.nih.gov/ij/) and three measurements per eggshell fragment were recorded. Locations of the three measurements on the images were chosen randomly.

Statistical analyses. Phylogeny, reproductive strategy, and body size can all influence eggshell thickness (Birchard and Deeming 2009). To help account for these sources of variability, we analyzed data for Palaeognathae and Neognathae taxa separately (Corfield et al. 2008).

We assessed agreement between micrometer- and SEM-based measurements using a tolerance-interval method (or the total deviation index method; Lin 2000, Choudhary 2008). Following Choudhary (2008), we first modeled the data using a linear mixed model and then constructed the relevant asymptotic tolerance interval for the distribution of appropriately defined differences. For each data set, consisting of repeated measurements using the two methods, we fitted a model that assumes random effects of the form $b_{ij} = b_i + b_{i*j}$, where b_i is the true unobservable measurement for the *i*th individual, b_{i*j} is the method-individual interaction, and (b_i, b_{i*1}, b_{i*2}) are mutually independent normal random variables with different variances (e.g., Bland and Altman 1999, Choudhary 2008). For the Neognathae, a random species effect was also included to account for the multiple individuals nested within each species. The maximum likelihood estimates of the model parameters were used to produce four estimates: (1) mean and standard deviation of the population of measurements for each method, (2) the intraclass correlations (relationship among measurements of a common class; for example, Lessells and Boag 1987) for each method, (3) correlation between the methods, and (4) the standard deviation of the population difference between any two measurements for a particular method.

The tolerance-interval approach focuses on differences between methods, and estimates the range (interval) of a specified proportion of the population of measurement differences. All the presented tolerance intervals assume 80% probability content (i.e., they cover 80% of the population of measurement differences) and a 95% confidence level (see Choudhary 2008). Tolerance intervals were calculated for both the intra- and intermethod agreement. In both cases, these intervals were contrasted with intervals constructed using a bootstrap-*t* approach (Choudhary 2008) that is recommended when the number of samples is ≤ 60. To determine if this agreement is sufficient, we can compare it with a threshold considered biologically meaningful. When such a threshold is not explicitly specified a priori, it is practical to compare the bound of the interval (U) with the magnitude of the measurements. If U is large relative to the magnitude (e.g., $>20\%$), we infer insufficient agreement; otherwise, sufficient agreement is inferred. The derivation of these statistics is provided by Choudhary (2008). All computational and data analyses were performed using the statistical software R (R Development Core Team 2009). Values are presented as means ± 1 SD.

RESULTS

We compared methods using eggshells from four groups of avian taxa (two intraspecific and two interspecific groups; Table 1, Fig. 1). Residual plots (Fig. 2) indicated that the model fits (Tables 2 and 3) were reasonable in all cases. As an example, we describe the kiwi data in detail. For the remaining data sets (cuckoo, Palaeognathae, and Neognathae) we refer to Table 1 and note similarities with the kiwi data.

For kiwi eggs (Fig. 1A), the estimated mean eggshell thickness was 311.83 ± 47.62 μm based on micrometer measurements and 323.17 ± 46.21 μm based on SEM measurements (Table 2). The estimated correlation between the two methods was 0.92 (Table 3).

Our estimate of the intraclass correlation was 0.96 for both the micrometer and SEM method (Table 2). The estimated standard deviation of the population difference was 13.07 for any two micrometer measurements and 12.71 for the SEM measurements (Table 2). Tolerance intervals for the intramethod agreement were ± 20.85 μm for the micrometer and ± 20.28 μm for the SEM. The bootstrap-*t* approach (Choudhary 2008) produced similar intervals (± 21.02 μm and ± 20.62 μm, respectively; Table 2). Thus, for repeated eggshell fragments from the same individual, 80% of both micrometer and SEM differences were estimated to lie within approximately ± 21 μm. These findings confirmed that both methods had good (and similar) repeatability.

We next considered agreement between the two methods. The mean difference between methods (micrometer minus SEM) was -11.34 ± 18.93 μm (Table 3). The tolerance interval for differences between the methods (micrometer minus SEM) was ± 36.01 μm.

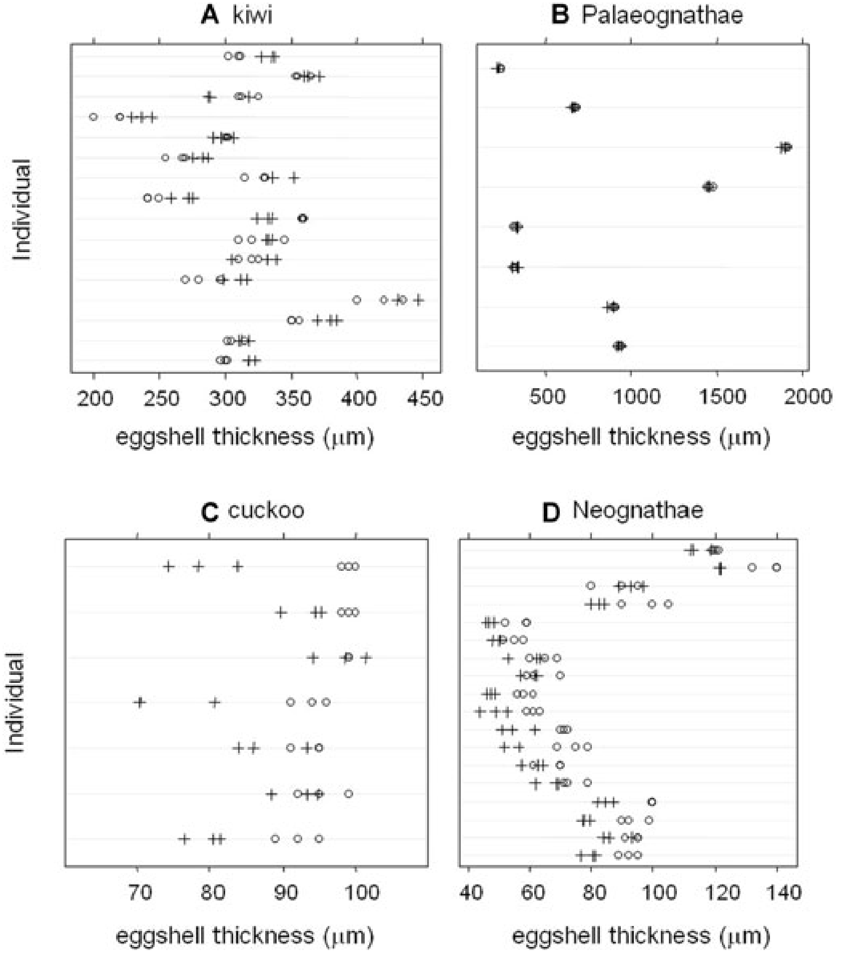

Fig. 1. Plots of eggshell thickness measurements (*x*-axis) for each eggshell sample (*y*-axis) from micrometer (o) and scanning electron microscope (+) methods for the four eggshell sample types; (A) kiwi, (B) Palaeognathae, (C) cuckoo, and (D) Neognathae. Data for each eggshell sample are plotted on different lines of the *y*-axis.

This interval was similar (±36.61 μm) using the bootstrap-*t* approach (Table 3).

For kiwi eggs ($N = 16$), the correlation between methods (0.92) was reasonably strong and, for repeated eggshell fragments from the same individual, 80% of the differences between methods (micrometer minus SEM) was estimated to lie within $U \pm 36$. For kiwi eggs, measurements ranged from 200 to 447 μm, and the bound ±36 is approximately 11% of the average measurement of about 320 μm, indicating reasonable agreement between micrometer and SEM measurements (Table 3).

The signs (directions) of the mean differences between micrometer and SEM measurements were not the same among the four taxonomic groups (Table 3). Measurements of eggshell thickness for kiwi eggs using the SEM were, on average, thicker than measurements using a micrometer. For the Palaeognathae, cuckoo, and Neognathae eggshells, micrometer measurements were, on average, thicker. In general, for samples with thicker shells (>300 μm; kiwi and Palaeognathae), both the micrometer and SEM methods had good, and comparable, repeatability (large intraclass correlation ≥0.96 and small intramethod difference; Table 2), and were of sufficient agreement (relative magnitude of intermethod agreement ≤12%; Table 3). However, for eggs with thinner shells (<100 μm;

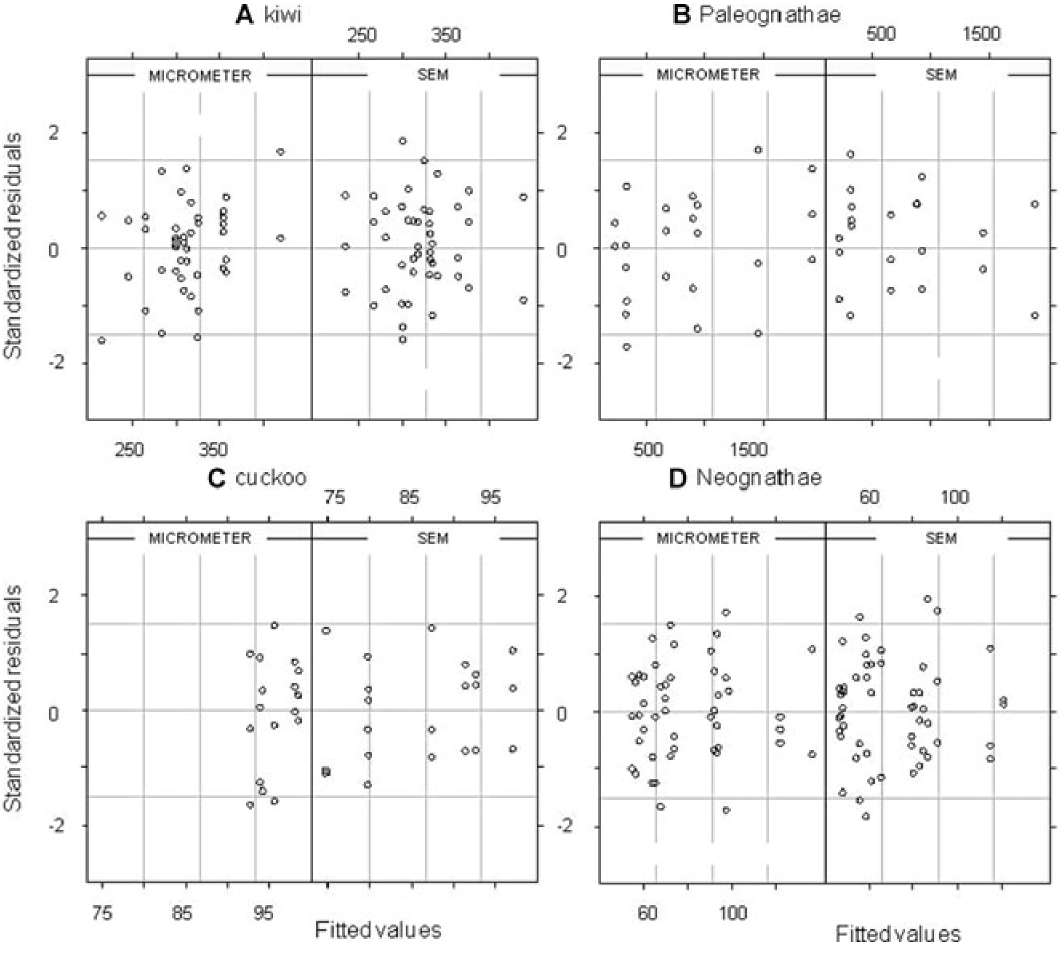

Fig. 2. Residual plots of the fitted model values (x-axis: μm) and residual values (y-axis) for each of the four eggshell sample types: (A) kiwi, (B) Palaeognathae, (C) cuckoo, and (D) Neognathae.

cuckoo and Neognathae), micrometer and SEM methods had relatively poor agreement (relative magnitude of intermethod agreement $\geq$26%; Table 3). The repeatability of the two methods for eggs with thinner shells, although worse than the repeatability in the samples with thicker shells, was not unreasonable because the relative magnitude of intramethod agreements was <11% (Table 2). However, the cuckoo eggs had lower intraclass correlations than those of the other taxonomic groups for both the SEM (0.76) and micrometer methods (0.55), and the correlation between methods was low (0.22). Based on differences in the size of the tolerance intervals, measurements of cuckoo eggs made with the micrometer clearly had greater repeatability than those made with the SEM.

DISCUSSION

Our results support the prediction of Booth and Seymour (1987) that eggshell thickness measured using micrometer and SEM techniques would yield statistically similar ordinal results. For all four of our taxonomic groups, measurements of eggshell thickness made using micrometer and SEM techniques yielded similar and reasonable intramethod repeatability based on the tolerance interval results. For kiwi and Palaeognathae eggs, the intermethod agreement was also reasonable.

However, SEM measurements were consistently smaller than micrometer measurements for cuckoo eggs. We measured the same eggshell samples using both methods, and measurements

Table 2. Estimates calculated from the model parameters for the intramethod level of agreement between micrometer (MICRO) and scanning electron microscopy (SEM) measurements of eggshell thickness. Results for the tolerance interval are based on an 80% probability content (for the proportion of the population of measurement differences) and 95% confidence level.

	N	Mean (SD) measurements		Intraclass correlation		SD population difference		Intramethod tolerance interval		Bootstrap-t interval	
		SEM	MICRO	SEM	MICRO	SEM	MICRO	SEM	MICRO	SEM	MICRO
Kiwi	16	323.17 (46.21)	311.83 (47.62)	0.96	0.96	12.71	13.07	±20.28	±20.85	±20.62	±21.02
Cuckoos	7	86.17 (8.50)	95.92 (3.40)	0.76	0.55	5.88	3.24	±10.87	±6.01	±11.12	±6.07
Palaeognathae	8	835.52 (550.78)	845.13 (550.80)	0.99	0.99	17.40	17.80	±31.13	±31.90	±31.99	±33.41
Neognathae	18	71.61 (22.15)	81.57 (22.54)	0.98	0.96	4.81	6.16	±7.57	±9.69	±7.75	±9.75

were made by the same individual, reducing the probability of a methodological or observer artifact in these variations. In fact, from Figure 2C, it is clear that the micrometer has good repeatability and its intramethod repeatability (tolerance interval) is better than that of the SEM. However, the intraclass correlation for the SEM is higher than for the micrometer (0.76 vs. 0.55). The explanation for the smaller correlation in the case of the micrometer, despite better repeatability, can be found from the plot of the data. From Figure 2C, it is clear that the ratio of between- and within-individual variation is only slightly higher than 1, suggesting that the intraclass correlation would be only slightly higher than 0.5 (if this ratio is 1, the intraclass correlation would be 0.5). It is also clear that this ratio for the SEM is larger than the ratio for the micrometer, indicating higher intraclass correlation for the SEM. The effect of between-individual variation on intraclass correlation has been noted previously (see Bland and Altman 1990).

One possible explanation for lower inter-method agreement for cuckoos and Neognathae than kiwis and other Palaeognathae is that eggs of the former taxa are (by an order of magnitude) smaller (at least for the species we selected). Measurements of smaller objects, where error is invariant to the size of the measurement, will result in proportionally larger errors than measurements of larger objects (Quinn and Keough 2002). We found that thinner eggshells were more difficult to measure with the micrometer than thicker eggshells. Similarly, thinner eggshell fragments were harder to mount for SEM photographs. The height at which fragments are mounted on the SEM stubs may also affect the accuracy of thickness measurements and this was also more difficult for smaller (thinner) eggshells.

Less likely is the possibility that eggshell thickness measures vary within the Neognathae, including cuckoos, because of some unique aspect of their eggshell structures. For example, egg maculation might affect repeated measurements from the same eggshell fragments. Gosler et al. (2005) showed that eggshell pigments in a small passerine (Great Tit, *Parus major*) were distributed nonrandomly with respect to eggshell thickness; protoporphyrin-based maculation was more dense in thinner shell regions. Thus, the presence or absence of maculation

Table 3. Estimates calculated from the model parameters for the intermethod level of agreement between micrometer and scanning electron microscopy (SEM) measurements of eggshell thickness. Results for the tolerance interval are based on an 80% probability content (for the proportion of the population of measurement differences; see Choudhary 2008) and 95% confidence level. The relative magnitude is the comparison of the intermethod tolerance bound with the average measurement (expressed as a percentage).

	N	Correlation between methods	Mean (SD) difference micrometer − SEM	Intermethod tolerance interval	Bootstrap-*t* interval	Relative magnitude (%)
Kiwi	16	0.92	−11.34 (18.93)	±36.01	±36.61	12%
Cuckoos	7	0.22	9.75 (8.44)	±24.37	±26.25	29%
Palaeognathae	8	0.99	9.60 (18.55)	±34.92	±34.74	4%
Neognathae	18	0.94	9.96 (7.57)	±19.36	±19.79	26%

on cuckoo eggs could have introduced variation into our measurements. Contrary to this possibility, half the species in our passerine samples within Neognathae had maculated eggs (both *Acrocephalus* spp. and Great Tit), with repeatability similar to what we found for our sample of Paleognathae taxa with immaculate eggs.

Overall, both micrometer and SEM methods performed similarly in measuring eggshell samples from a variety of taxa. We conclude that the more time- and cost-effective method of using a micrometer is no less reliable than using the SEM technique. In addition, although potentially yielding additional insights and detailed analyses into the structure and elemental composition of eggshells (Blankespoor et al. 1982, Booth and Seymour 1987, Nys et al. 2004), microscopy also represents a semidestructive technique, requiring preparation of the samples, for example, coating with a heavy metal, that may preclude returning the samples to museum collections and interfere with additional structural and compositional analyses of the same shell fragments. Such considerations represent a further set of trade-offs in feasibility and access for one of the methods tested in our study, especially for rare or irreplaceable samples, including eggshells of endangered or extinct species (Igic et al. 2010).

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