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# Eggshell Penetration of Various Types of Hens' Eggs by *Salmonella enterica* Serovar Enteritidis

**WINY MESSENS,[1]* KOEN GRIJSPEERDT,[1] KOEN DE REU,[1] BART DE KETELAERE,[2] KRISTOF MERTENS,[2] FLIP BAMELIS,[2] BART KEMPS,[2] JOSSE DE BAERDEMAEKER,[2] EDDY DECUYPERE,[2] AND LIEVE HERMAN[1]**

[1]*Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Unit, Brusselsesteenweg 370, B-9090 Melle, Belgium; and* [2]*Egg Quality and Incubation Research Group, Department of Biosystems, Katholieke Universiteit Leuven, Kasteelpark Arenberg 30, B-3001 Leuven, Belgium*

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## ABSTRACT

Egg weight, shell thickness, number of pores, cuticle deposition, eggshell strength (dynamic stiffness and damping ratio), and the ability of *Salmonella enterica* serovar Enteritidis (SE) to penetrate the eggshell were determined. Penetration was assessed by filling the eggs with a selective medium that allowed viewing of *Salmonella* growth on the inside of the shell and membrane complex. After inoculation of each shell with on average 2.71 log CFU, the eggs were stored for up to 14 days at 20°C and 60% relative humidity. Commercially available eggs were used. At 14 days of storage, only 6.0% of the eggs from free-range hens and 16.0% of the generic (i.e., eggs from hens in conventional battery cages that were given standard feed) white eggs were penetrated. The generic brown, organic, and omega-3–enriched eggs were penetrated at a frequency of 30 to 34%. In a second experiment it was shown that the layer strains of the hen (ISA-Brown Warren versus Bovans Goldline), which were kept in furnished cages, did not affect eggshell penetration by SE. For Bovans Goldline hens, the housing system (furnished cage versus aviary) did not affect penetration, while a trend was visible toward a higher fraction of penetrated eggshells when hens were fed corncob mix rather than standard feed. Eggshell penetration was observed more frequently in the absence of cuticle spots and for eggs having lower dynamic stiffness values. Shell contamination at the end of storage was highly correlated with SE penetration.

Egg-associated infections are mainly caused by *Salmonella enterica* serovar Enteritidis (SE), and eggshells are considered the predominant source of human salmonellosis in Europe. Of the 145,000 reported cases in 2002, about 70% were caused by SE *(11).* Intact eggs can become contaminated with SE as a result of infections of the reproductive tissues of the laying hens and by penetration through the eggshell. The first is considered the dominant route of infection *(4, 30).* Shell contamination is the first requisite for penetration, and contamination can occur with any organism that is excreted by the laying hen and by contact with nesting material, dust, and feed *(25).*

The dominant system for the production of eggs in the EU is the conventional battery cage (CC), which will be prohibited starting from 1 January 2012 *(23).* CC production still accounts for 85% of egg production across the EU (apart from Sweden) *(12).* The use of cages improved by environmental enrichments (so-called furnished cage [FC] system) is still marginal. Alternative or noncage (NC) systems are used in the form of free-range and barn systems *(23).* In general, bacterial contamination seems to be slightly but significantly higher on shells from eggs laid in FCs than in CCs *(8, 22, 32).* In NC systems, this level is even higher *(8, 29)* and seems to be related mainly to a higher microbial load of the environment of the laying house. In theory, the risk of contamination with *Salmonella* and particularly with SE might be higher when eggs are produced in some NC systems, because of the greater exposure of layers and their eggs to environmental contamination *(10).* However, a study by the United Kingdom Food Standards Agency *(13)* did not find significant differences in *Salmonella* contamination of the shell at the retail level, due to production systems.

As the shell structure of eggs produced in NCs has been found to be inferior to that of eggs from cages *(14, 15),* it has been hypothesized that this defect could impair their ability to stop the penetration by SE of eggs *(15).* Up to now, very little research has assessed the influence of layer strain and housing system or feed on eggshell penetration. This research is, however, important, especially at this moment with the move to welfare-friendlier systems. Besides studying the presence and levels of eggshell contamination in the different systems, one should also examine the possibility of eggshell penetration.

Previous studies by our group on one test flock of ISA-Brown Warren hens housed in CCs indicated that neither flock age *(7, 9, 24)* nor storage conditions *(26)* significantly influenced eggshell penetration by SE. Hen age affected all shell characteristics studied, but these shell characteristics could not be correlated with penetration *(24).* The present study was set up to include commercially available eggs having a broader range of eggshell characteristics and to study the influence of genetic strain of hen, feed, and holding system on eggshell penetration by SE.

* Author for correspondence. Tel: 0032-9-2723000; Fax: 0032-9-2723001; E-mail: winy.messens@ilvo.vlaanderen.be.

TABLE 1. *Eggs and laying hens used in experiment 1*

| Type of egg | Genetic strain of the hen | Hen's age (no. of wk) | Housing system | Feed |
|---|---|---|---|---|
| Free-range | Bovans | 39 | Free-range | Supplemented |
| Generic brown | ISA-Brown Warren | 63 | CC | Standard |
| Generic white | Lohmann Selected Leghorn (LSL) | 88[a] | CC | Standard |
| Omega-3-enriched | ISA-Brown Warren | 21 | CC | Balanced |
| Organic | Shaver | 32 | Free-range | Organic |

[a] Hens were molted at 70 weeks of age.

TABLE 2. *Eggs and laying hens used in experiment 2*

| Code | Genetic strain of the hen | Housing system | Feed |
|---|---|---|---|
| IFS | ISA-Brown Warren | FC | Standard |
| BFS | Bovans Goldline | FC | Standard |
| BAC | Bovans Goldline | Aviary | CCM |
| BAS | Bovans Goldline | Aviary | Standard |

## MATERIALS AND METHODS

**Eggs and laying hens.** For this study only intact eggs, as assessed by candling and vibration experiments (see further), without visible fecal or egg content contamination of the shell were used. In experiment 1, various types of eggs that are commercially available were collected (Table 1). Eggs were 7 to 9 days postlay at the start of the study. Generic eggs are defined as eggs from CC by using standard feed. The feed of the free-range hens was supplemented with oyster shells. Omega-3 fatty acid content in the yolks of omega-3–enriched eggs is increased by using pulses, greens, and oil seeds to enrich the foundation of wheat and soy. Doing so achieves a nearly perfect 1:1 balance between omega-6 and omega-3 fatty acids in the egg and a higher vitamin E content. Hens laying organic eggs are given a mixture of seeds and grains, pulses and oil-containing crops, and a supplement of calcium; about 80% of the feed is organic.

In experiment 2, eggs of two commercially available genetic strains of laying hens (ISA-Brown Warren versus Bovans Goldline) kept in two types of housing systems (FC versus aviary) and fed two types of feed (standard feed versus feed containing corncob mix [CCM]; both types of feed had equivalent nutritional composition), were collected at the Provincial Centre for Applied Poultry Research (Geel, Belgium) (Table 2). Hens were 25 weeks of age. The FCs housed 43 birds, each cage (2.4 m by 1.1 m) having a nest box of 0.33 $m^2$. Hens were fed ad libitum by means of linear troughs. Perch length per hen was 15 cm. The aviary had two levels of wooden platforms and nonintegrated nest boxes. The group size was 500 birds. Feed was administered ad libitum by circular troughs. The eggs were stored overnight at 20°C until use and were only 24 h postlay at the start of the study.

**Bacterial strain and cultures.** SE MB1409 was used, a strain that was isolated from egg contents and made resistant to streptomycin via natural selection by exposure to 25 mg (ppm) of streptomycin per liter. The strain was freshly propagated. Next, one colony was picked and grown overnight at 37°C in 9 ml of buffered peptone water (Oxoid, Basingstoke, UK) containing 25 mg (ppm) of streptomycin per liter and were diluted with phosphate-buffered saline (PBS; Oxoid) until $10^{-2}$ dilution. The counts of viable SE cells in this immersion suspension were $1.1 \times 10^6$ CFU/ml for the first experiment and $3.9 \times 10^6$ CFU/ml for the second experiment. Enumeration was done by serial decimal dilution, plating out on tryptone soy agar (Oxoid) and incubation overnight at 37°C.

**Agar molding technique.** An agar molding technique was used to view bacterial penetration of the eggshell as described in detail by Messens et al. *(24).* This method consisted of sucking out the egg contents, removing the albumen adhering to the membranes by washing with 1/4-strength Ringer solution (Oxoid), and filling the egg with sterile molten (50°C) plate count agar (Oxoid) containing 25 mg (ppm) of streptomycin/liter and 1 g of the indicator 2,3,5-triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, Bornem, Belgium) per liter. Addition of streptomycin ensured that only the inoculated streptomycin-resistant SE was able to grow, thus inhibiting competitors. When bacterial penetration of the eggshell occurred, SE grew on the agar and reduced the TTC to formazan (with redness). Penetration was recorded when red colonies were visible by candling. Candling was done carefully, preventing cross-contamination, daily during the first week and three times a week later. *Salmonella*'s presence was confirmed by swabbing at the red spots with a sterile applicator to transfer the bacteria to a plate of xylose lysine desoxycholate (XLD; Oxoid) agar.

**Inoculation and storage.** For each experimental set of conditions, 50 agar-filled eggs and 20 whole eggs were exposed to the streptomycin-resistant SE MB1409 by dipping for 1 min in the immersion suspension. Both the eggs and the immersion solution were at 20°C. All eggs were kept under ambient conditions until dry. Afterward, the agar-filled eggs were placed in a climate chamber at 20°C and 60% relative humidity (KBP 6395 F, Termaks; Solheimsviken, Norway) for up to 14 days, while the whole eggs were immediately used for determination of inoculation dose, i.e., the contamination with *Salmonella* of shells immediately after inoculation.

**Determination of eggshell characteristics.** The dynamic stiffness ($K_{dyn}$) and damping ratio were measured immediately before candling, i.e., before the penetration experiment, by acoustic resonance frequency analysis relying on the lab-scale test arrangement described by De Ketelaere et al. *(6).* Details relating to the calculation of $K_{dyn}$ and the damping ratio can be found in Coucke *(5)* and De Ketelaere et al. *(6).* The egg weight ($W$) of the fresh eggs was measured, and the corresponding formula $S = 4.67 \times W^{2/3}$ was used to calculate the shell area *(33).*

Upon completion of the penetration experiment, the contamination by *Salmonella* of all eggshells was quantified, and afterward the cuticle score, number of pores, and shell thickness were analyzed on 20 agar-filled eggs, randomly selected from a total of 50 eggs used under each set of conditions. The analysis of the cuticle consisted of dying with an aqueous solution containing per liter 7.2 g of Tartrazine and 2.8 g of Green S (Barentz NV, Zaventem, Belgium) (method developed by Board and Halls *(3)*). The remaining redness, i.e., the shell color at places to which the dye did not bind, was analyzed with Paint Shop Pro version 8 (Jasc Software, Eden Prairie, Minn.) by using the histogram function. To calculate the cuticle score, the red value score was subtracted from 255, i.e., the maximum value in the redness plane. Almost no cuticle is deposited on the shell when the cuticle score is <110 and when tiny cuticle spots are observed between scores of 110 and 140. The number of pores was determined by using a method described by Tyler *(33)* of microscopic counting (eyepiece, ×10; objective, ×10) (Diaplan type 307-148.001; Leitz, Wetzlar, Germany) after immersion of pieces of shell taken from the entire

TABLE 3. *Egg(shell) characteristics of various commercially available eggs used in experiment 1*[a]

| Type of egg | Egg wt (g)[b] | Shell thickness (μm)[c] | Cuticle score[c] | No. of pores/shell[c] | Dynamic stiffness ($K_{dyn}$, N/m)[b] | Damping ratio (%)[b] | Contamination by *Salmonella* of shells (log CFU/shell)[b] |
|---|---|---|---|---|---|---|---|
| Free-range | 66.7 ± 2.6 C | 383 ± 22 A | 186 ± 22 A | 1,500 ± 810 A | 15,000 ± 1,500 B | 2.88 ± 0.73 A | 0.12 ± 0.50 A |
| Generic brown | 66.5 ± 2.4 C | 408 ± 26 B | 141 ± 38 B | 1,400 ± 1,100 A | 16,000 ± 1,900 C | 2.85 ± 0.60 B | 0.86 ± 1.53 B |
| Generic white | 69.5 ± 4.1 D | 371 ± 31 A | 132 ± 49 B | 1,500 ± 1,100 A | 16,500 ± 1,600 C | 2.38 ± 0.45 A | 0.69 ± 1.73 B |
| Omega-3–enriched | 63.3 ± 2.5 B | 414 ± 15 B | 174 ± 29 A | 1,500 ± 790 A | 14,100 ± 1,500 A | 3.77 ± 1.11 C | 0.80 ± 1.62 B |
| Organic | 60.4 ± 3.6 A | 402 ± 26 B | 179 ± 32 A | 1,100 ± 550 A | 14,900 ± 2,400 B | 3.63 ± 1.20 C | 0.77 ± 1.37 B |
| | ***[d] | *** | *** | NS[e] | *** | *** | *[f] |

[a] Values are means ± standard deviation. Means in a column followed by identical letters are not significantly different ($P > 0.05$, Duncan's test).
[b] Fifty eggs sampled.
[c] Twenty eggs sampled.
[d] $P < 0.001$.
[e] Not significant.
[f] $P < 0.05$.

egg for 25 s in 65% nitric acid, rinsing with distilled water, and removal of the membranes. The number of pores visible on each area of focus (approximately 3 $mm^2$) was counted on 20 areas and was expressed as number of pores per shell. The shell thickness with the shell membranes attached was determined with a micrometer on three places at the equator. The mean value was used for calculations.

**Determination of contamination with *Salmonella* of eggshells.** Ten milliliters of PBS (Oxoid; at room temperature) was added to one egg in a plastic bag, and the eggshell was rubbed through the bag for 1 min to detach the bacteria. Enumeration of *Salmonella* was done by surface plating 1 ml of the diluent on XLD (Oxoid) agar in a large petri dish. In case of high counts on these large plates, serial decimal dilutions of the diluent (stored refrigerated overnight) were plated on XLD (Oxoid) agar. In case of no counts, the remainder of the diluent was enriched with buffered peptone water (Oxoid) at a ratio of 1:3 (vol/vol). After incubation overnight at 37°C, a loopful was streaked on an XLD plate. Plate contents were incubated at 37°C for 24 h. For calculation, counts of 5 CFU per eggshell (or per 10 ml of PBS solution) were given upon observation of no growth after plating 1 ml of diluent but visible growth after enrichment. To allow a log transformation, counts of 1 CFU per eggshell were given upon observation of no colonies after enrichment.

**Statistical analysis.** One-way analysis of variance was used to study the effect of type of egg on shell characteristics. Significant differences were assessed by Duncan's post hoc test. A $P$ of $<0.05$ was considered to be statistically significant. Noncentral confidence interval (CI) estimation was used to calculate the CIs at a 95% confidence level of the percentage of penetrated eggshells of the various eggs. The penetration data were analyzed by using a generalized linear regression with penetration at the end of storage (yes/no) as a binomial-dependent variable and the eggshell characteristics and log-transformed *Salmonella* surface counts as continuous independent variables. A logit link function was used to relate the continuous predictors and binomially distributed dependent variable. All analyses were done in Statistica 6.1 (Statsoft Inc., Tulsa, Okla.).

## RESULTS AND DISCUSSION

Egg(shell) characteristics of the commercially available eggs are shown in Table 3. The mean weight of the generic white eggs that are laid by the oldest hens was highest. Thinnest eggshells were from eggs from free-range hens and generic white eggs. Overall, shell thickness ranged between 323 and 443 μm. Cuticle deposition varied strongly, but the mean deposition of the generic white and generic brown eggs was lowest. Almost no cuticle was observed on the shell (6 of 20 generic white eggs and 5 of 20 generic brown eggs), or only tiny cuticle spots (4 of 20 generic white eggs and 5 of 20 generic brown eggs) were present on 50% of these eggs. For eggs from free-range hens and organic and omega-3–enriched eggs, this property was observed on 0, 10, and 5% of the eggshells. The mean number of pores per shell was not significantly different between the groups and overall varied between 55 and 4,953 pores. Pores were distributed as clusters, but the mean number of pores was higher at the blunt pole than at the apex. Mean dynamic stiffness was higher for generic brown and generic white eggs than for all others, while mean damping ratio was lowest for generic white eggs.

In experiment 2, shell thickness varied between 323 and 483 μm, and the number of pores ranged between no pores observed in the field of view (on 60 $mm^2$ of shell) and 1,964 pores per shell. Tiny cuticle spots were observed on only one egg out of 80. The mean number of pores was higher for eggs from Isa-Brown Warren hens and for eggs from Bovans Goldline hens given CCM (Table 4). The damping ratio was slightly affected.

Considering both experiments, the mean initial contamination dose was 2.71 ± 0.40 log CFU per eggshell. The first appearance of penetration was mostly visible on day 3. At day 5 more than 95% eggshell penetration was observed, if one took into account the eggs that became penetrated. In the present study, the generic brown eggs had a penetration percentage of 30%, not statistically different from the organic and omega-3–enriched eggs having a penetration percentage of 34%. The eggs from free-range hens and generic white eggs, however, were better at resisting penetration: only 6% of the former and 16% of the latter became penetrated (Fig. 1a). These generic white eggs were

TABLE 4. *Egg(shell) characteristics of eggs used in experiment 2*[a]

| Type of egg[b] | Egg weight (g)[c] | Shell thickness (μm)[d] | Cuticle score[d] | No. of pores/shell[d] | Dynamic stiffness ($K_{dyn}$, N/m)[c] | Damping ratio (%)[c] | Contamination by *Salmonella* of shells (log CFU/shell)[c] |
|---|---|---|---|---|---|---|---|
| IFS | 55.6 ± 4.3 A | 409 ± 39 A | 167 ± 20 A | 710 ± 500 B | 13,000 ± 1,700 A | 3.77 ± 1.14 B | 1.64 ± 2.22 A |
| BFS | 55.6 ± 3.1 A | 393 ± 24 A | 180 ± 20 A | 330 ± 250 A | 13,100 ± 1,700 A | 3.48 ± 1.04 AB | 1.52 ± 2.07 A |
| BAC | 55.6 ± 3.7 A | 404 ± 27 A | 178 ± 13 A | 580 ± 360 B | 14,000 ± 1,900 A | 3.07 ± 0.93 A | 2.37 ± 2.11 A |
| BAS | 56.7 ± 4.1 A | 405 ± 21 A | 180 ± 15 A | 340 ± 310 A | 13,300 ± 1,400 A | 3.32 ± 0.93 A | 1.50 ± 2.10 A |
| | NS[e] | NS | NS | **[f] | NS | ** | NS |

[a] Values are means ± standard deviation. Means in a column followed by identical letters are not significantly different ($P > 0.05$, Duncan's test).
[b] For description see Table 2.
[c] Fifty eggs sampled.
[d] Twenty eggs sampled.
[e] Not significant.
[f] $P < 0.01$.

from old hens (88 weeks), indicating reduced eggshell penetration for older flocks. A trend toward reduced SE penetration of eggshells with increasing flock age was found by our group before: 45% penetration at the beginning of lay versus 31.6% at the end in one study *(24)* and 46 versus 29% in a second study *(7)*. De Ketelaere et al. *(6)* has already indicated that changes occur in eggshell structure during ageing of the hen, as shell thickness decreased during the laying period without producing weaker eggs. Between eggs from free-range hens and organic eggs also originating from free-range hens, significantly different penetration rates were found.

It was observed that the eggs with penetration percentages ranging between 30 and 45% (generic brown, organic, and omega-3–enriched eggs) were from ISA-Brown Warren or Shaver hens. The generic white eggs and eggs from free-range hens were from LSL and Bovans hens, respectively. Because this experiment was not standardized (hen ages differed, molting occurred for hens laying the generic white eggs, and feed differed [Table 1]), definite conclusions cannot be drawn and a second experiment was conducted. This experiment (Table 2 and Fig. 1b) included eggs from both ISA-Brown Warren and Bovans Goldline hens kept in two types of housing systems (FC versus aviary) and fed two types of feed (standard feed versus feed containing CCM). The genetic strain of the hens (kept in FCs and given standard feed) did not significantly affect the eggshell penetration by SE, as 44% of the eggs from ISA-Brown Warren hens and 42% of the eggs from Bovans Goldline hens were penetrated. Both flocks were from commercial layer strains. Upon comparison of eggs of layer birds (9.7%) and broiler breeders (16.1%), significant differences were observed by others *(31)*. Eggs from an Ottawa control strain and a current commercial stock were

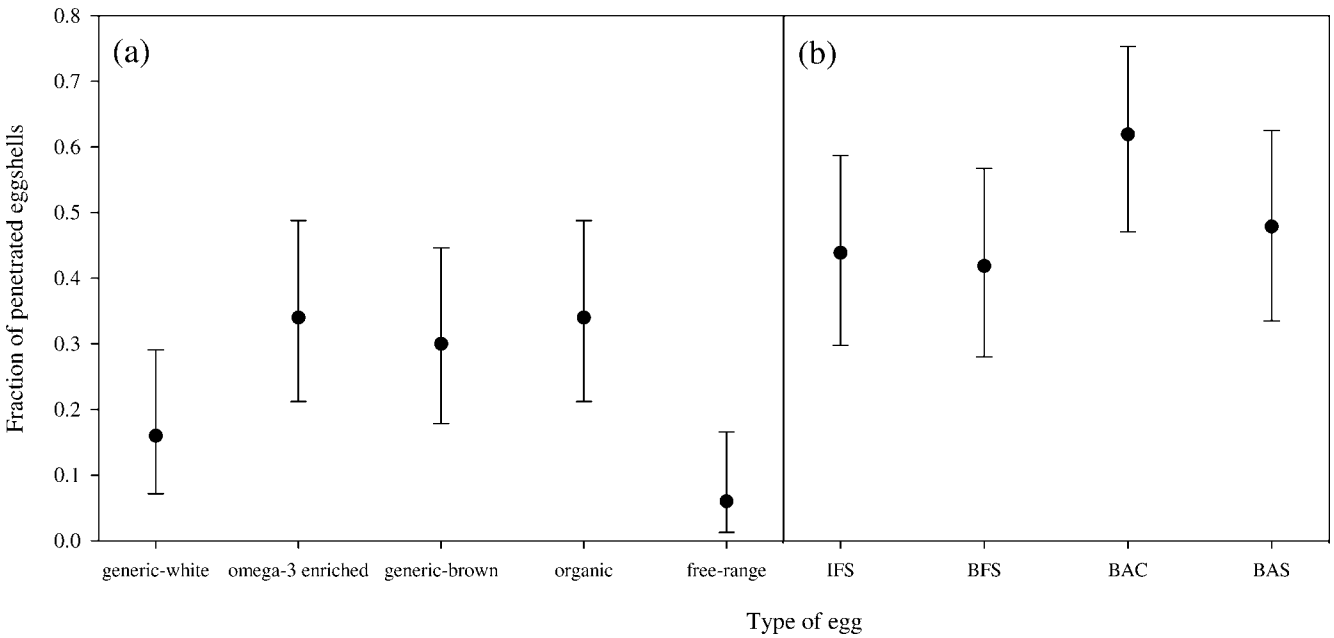


FIGURE 1. *Fraction of eggshells penetrated by* Salmonella *after 14 days of storage at 20°C and 60% relative humidity as a function of type of egg in experiments 1 (a) and 2 (b). Vertical bars represent 95% CIs; the number of eggs sampled* (n) *was 50 in each group.*

found more easily contaminated than the other two control strains *(19, 20).* When hens were housed in an aviary, 48% of the eggs from Bovans Goldline hens were penetrated. For these hens, the housing system (FC versus aviary) did not give significantly different penetration probabilities. As only intact eggs were used in our study, eggs with hairline cracks, a defect that is noted when eggs are candled, were removed. Several authors have reported significant increases of this defect in eggs collected in FCs *(1, 16, 22, 34)* depending on the design of these cages *(17, 35).* This defect will give rise to very frequent eggshell penetration (13 eggshells were penetrated of a total of 14 eggshells with cracks, i.e., 93% [unpublished results]). A trend was visible for the feed: eggs from Bovans Goldline hens kept in aviaries and fed CCM were more frequently penetrated (62%) than were eggs of hens given standard feed (48%) (although not significant: $P = 0.16$). In this experiment it should be noted that the egg weight, shell thickness, and cuticle score of the eggs collected were not affected by the genetic strain of the hen, housing system, or feed. Mallet et al. *(22)* also observed that cage type (FC versus CC) had no effect on eggshell breaking strength and shell thickness.

Evaluation of data from both experiments showed no significant ($P > 0.05$) difference between them in shell area, shell thickness, number of pores, damping ratio, and the presence or absence of SE eggshell penetration. This has been observed before for pores *(9, 18, 24, 28)* and shell thickness *(9, 24).* Higher ($P < 0.0001$) contamination with *Salmonella* of shells at 14 days of storage was found for the eggshells that were penetrated than for those that were not penetrated (Fig. 2a) as observed in other studies *(7, 9, 24, 26).* The mean value of $K_{dyn}$ ($P = 0.0066$) was lower for the penetrated than for the unpenetrated eggshells (Fig. 2b). Dynamic shell stiffness that allows a rapid assessment of shell strength *(2)* can thus provide information on possibility of eggshell penetration as shown in our study. Jones and Musgrove *(21)* recently reported that eggshell strength does not play a major role in SE contamination. In their study, eggshell strength was determined with a quasistatic compression test, and penetration was assessed on whole eggs. The mean value of cuticle deposition ($P = 0.0037$) was lower for penetrated than for unpenetrated eggs (Fig. 2c), indicating that cuticle deposition is important for the prevention of eggshell penetration. It is noted especially that, in the absence of cuticle, eggshell penetration is a very frequent event, as 10 of 13 eggshells that lacked cuticle were penetrated. The amount of cuticle deposited was not related to eggshell penetration in our previous study *(24)*; however, in the absence of cuticle there were also 9 out of 12 eggshells penetrated. It thus seems that especially in the absence of cuticle eggshell penetration is enhanced, but when cuticle is present, its degree does not affect shell penetration. This finding is important, as it was observed that the cuticle deposition of the generic eggs was bad for half of the eggs. The diversity of the cuticular coverage has been observed before and confirmed as an infrequent accessory rather than an inherent aspect of the true shell by Watt *(36)* and Nascimento *(27).* Also, prior egg handling could have caused the removal of cuticle.

To conclude, the present study indicates that huge var-

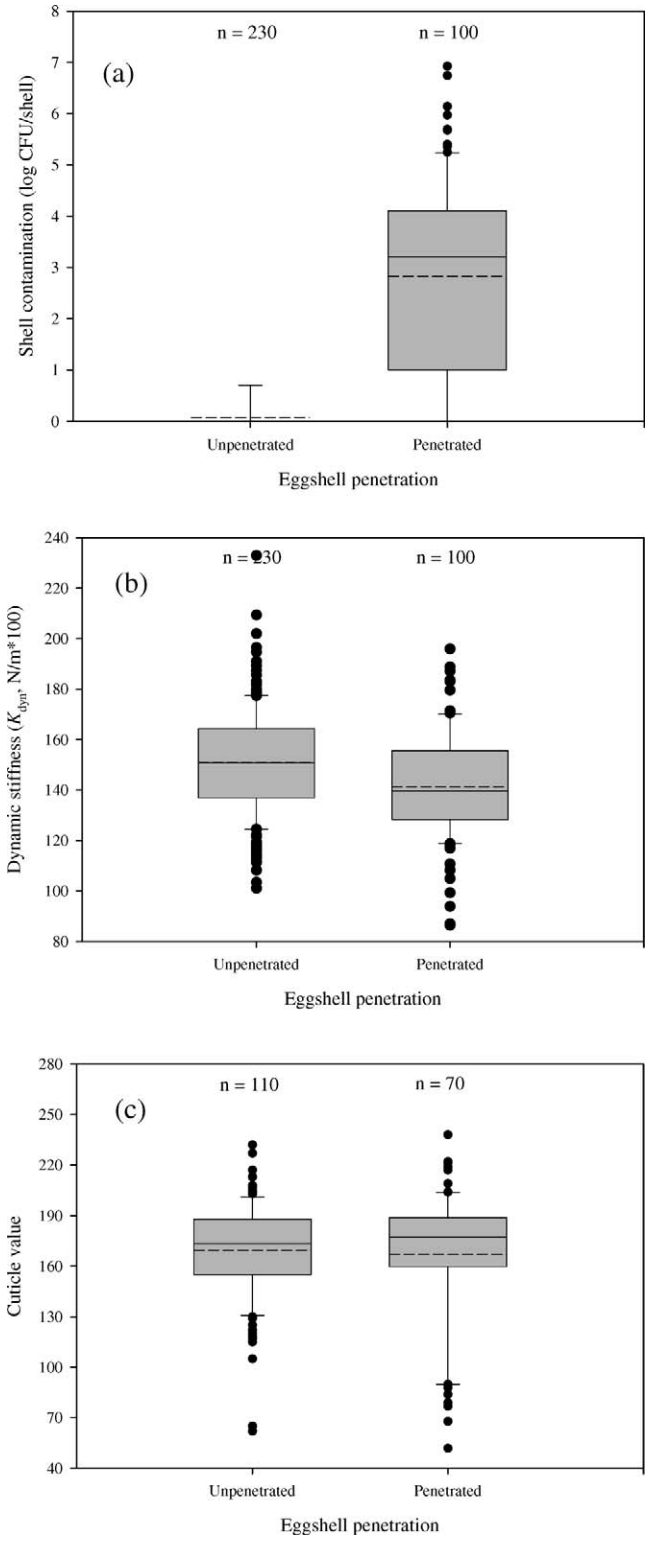


FIGURE 2. *Shell contamination (a), dynamic stiffness (b), and cuticle value (c) of unpenetrated versus penetrated eggshells. The number of eggs sampled* (n) *is shown. The solid line within the box marks the median, while the dashed line marks the mean. The boundaries of the box represent the 25th and 75th percentiles. Whiskers above and below the box indicate the 10th and 90th percentiles. The outliers, i.e., all data points that lie outside the 10th and 90th percentiles, are shown as symbols.*

iations exist in the ability of eggshells to resist penetration. This discrepancy could not, however, be traced back to the genetic strain of the laying hen or housing system. The feed administered to the laying hens might help aid to prevent eggshell penetration, but further research is needed to confirm this theory. Although shell contamination was highly correlated with SE penetration, penetration was observed more frequently in the absence of cuticle spots and for eggs having lower dynamic stiffness values.

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