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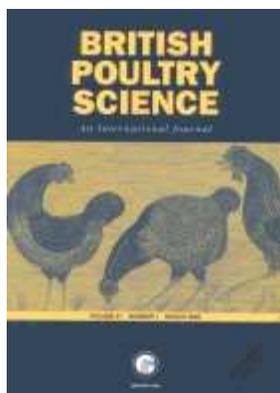
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Eggshell penetration of hen's eggs by *Salmonella enterica* serovar Enteritidis upon various storage conditions

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1

2 **Eggshell penetration of hen's eggs by *Salmonella enterica* serovar**3 **Enteritidis upon various storage conditions**

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9 Running title: *Salmonella* penetration and egg storage

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1 **Abstract** 1. The survival and penetration of *Salmonella enterica* serovar
2 Enteritidis (SE) inoculated on the eggshell was examined upon storage for up to 20 d
3 at real-life conditions (15°C to 25°C and 45% to 75% relative humidity (RH)).
4 2. Penetration was assessed by emptying the egg contents and filling the eggs with a
5 selective medium that allowed visualising *Salmonella* growth on the inside of the
6 shell and membrane complex.
7 3. The study of survival on the eggshells was based on viable counts and showed
8 that numbers of surviving organisms decreased over time. Survival was inversely
9 related to storage temperature and RH. Although the average counts decreased over
10 time, a limited proportion of shells carried high numbers of SE at all storage
11 conditions.
12 4. Penetration spots were observed earlier using an increased storage temperature
13 due to increased growth rates of SE on the agar. After 20 d of storage a similar
14 percentage (ca. 44.7%) of eggshells became penetrated, irrespective of the storage
15 conditions tested in this study.
16 5. The higher the *Salmonella* shell contamination at the end of storage, the higher
17 the probability that the eggshell was penetrated. *Salmonella* shell counts exceeding 4
18 log cfu yielded more than a 90% probability of eggshell penetration occurring.

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INTRODUCTION

2 Eggshells can be penetrated by various bacteria, among which is *Salmonella enterica*
3 serovar Enteritidis (SE). This serotype is linked with human salmonellosis cases
4 caused by the consumption of shell eggs and egg products. In Belgium, in 2004, 9
5 543 cases were reported, of which 63.6% were caused by serovar Enteritidis (Collard
6 and Bertrand, 2005). Various studies have been conducted to examine the probability
7 of *Salmonella* penetration through the eggshell. The major extrinsic factors identified
8 as being important to the transmission of *Salmonella* through the shell are: bacterial
9 strain, temperature differential, moisture, number of organisms present on the
10 eggshell, and storage conditions. The most important intrinsic factor affecting SE
11 penetration is the presence of shell defects, such as cracks (Messens *et al.*, 2005b).

12 The process responsible for eggshell contamination by infected birds is not yet
13 clear. Shell contamination most likely depends on either intestinal and oviduct
14 infection. The egg surface can be contaminated with faeces containing *Salmonella*
15 during expulsion of the egg from the hen, that is, intestinal infection (Gast and Beard,
16 1990). The egg surface can also be contaminated within the hen reproductive system
17 after formation of the shell, that is, oviduct contamination (Humphrey *et al.*, 1991).
18 Following oviposition, the shell acquires contamination from all surfaces with which
19 it makes contact and the extent of contamination is directly related to the cleanliness
20 of these surfaces (Board and Tranter, 1995). Contamination in egg-packing plants
21 may also be a significant contributory factor to external contamination of shell eggs
22 by *Salmonella* (Davies and Breslin, 2000a) and other bacteria (De Reu *et al.*, 2005).

23 *Salmonella* organisms on eggshells can die rapidly during storage but survival
24 is enhanced by low temperatures (Rizk *et al.*, 1966; Baker, 1990; Braun *et al.*, 1999;
25 Radkowski, 2002), especially when relative humidities (RHs) are low (Simmons *et*

1 *al.*, 1970). *Salmonellae* probably survive for a longer time under lower temperature
2 due to the slower metabolism induced by the disadvantageous conditions on the dry
3 eggshell surface (Radkowski, 2002). The presence of *Salmonella* on the surface of
4 the shell can give rise to cross contamination in the kitchen and to eggshell
5 penetration.

6 This study was designed to examine the effect of storage conditions on
7 survival/growth of SE on the eggshell and on the ability of SE to penetrate the shell.
8 The storage conditions were specified in such a way to resemble the environment the
9 eggs may be exposed to in Western Europe: in the egg-laying farm in summer
10 (25°C–75% RH) or winter (15°C–75% RH), in the egg-packing plant (15°C–45% RH
11 to 20°C–60% RH) and in the store in the summer (25°C–45% RH) or winter (15°C–
12 75% RH) (personal communication with François Porin, INRA-LORIA, Ivry sur
13 Seine, France).

14 MATERIALS AND METHODS

15 Eggs

16 Eggs from ISA-Brown Warren hens maintained in conventional cages were obtained
17 from a test flock at the Department of Animal Nutrition and Husbandry, section
18 Small Stock Husbandry (Agricultural Research Centre-Ghent, Belgium). Eggs were
19 collected in the period January-June 2003 at the middle (34-51 weeks of hen age) of
20 lay. No egg out of 30 eggs sampled tested positively for antibiotics (Premi[®]Test-Egg
21 kit) or *Salmonella* antibodies (Desmidt *et al.*, 1996). Only intact eggs assessed by
22 candling without visual faecal or egg contents contamination of the shell were taken.
23 The eggs were stored overnight at 20°C until use and were only 24 h post lay at the
24 start of the study.

1 **Bacterial strain and cultures**

2 *S. Enteritidis* MB1409 was used, a strain that was isolated at our laboratory from egg
3 contents and made resistant to streptomycin. Bacteria were resuscitated, then plated
4 and incubated at 37°C as previously described (Messens *et al.*, 2005a). Next, one
5 colony was grown overnight at 37°C in 9 ml BPW containing 25 ppm streptomycin
6 and diluted (to 10⁻²) with phosphate buffered saline (PBS; Oxoid, Basingstoke, UK).
7 The count of viable SE cells in this immersion solution was 5.3×10⁶ colony-forming
8 units (cfu)/ml. Enumeration was done by serial decimal dilution as described
9 (Messens *et al.*, 2005a).

10 **Agar moulding technique**

11 An agar moulding technique was used (Messens *et al.*, 2005a). In which the egg
12 contents were replaced by sterile molten (50°C) PCA (Oxoid) containing 25 ppm
13 streptomycin and 1 g/l 2,3,5-triphenyl tetrazolium chloride (TTC; Sigma-Aldrich,
14 Bornem, Belgium). Adding streptomycin to the agar ensured that only resistant
15 bacteria could grow, thus inhibiting competitors of the resistant SE. TTC is reduced
16 by growing bacteria, resulting in dark red colours. At the end of the experiment, all
17 agar-moulded eggs were aseptically opened. On some eggs the inside of the inner
18 membrane was swabbed at the red spots with a sterile cotton-tipped applicator. The
19 applicator was used to transfer the bacteria to a plate of xylose lysine desoxycholate
20 (XLD; Oxoid) agar and incubation for 24 h at 37°C. Growth of *Salmonella* was
21 obvious on the plates.

22 The agar-filled eggs were inoculated and placed in the climate chamber.
23 Penetration was recorded as the number of red colonies visible on the agar when the
24 agar-filled eggs were candled carefully, preventing cross-contamination, daily during
25 the first week and three times a week later. A TSA (Oxoid) plate was inoculated with

1 the SE strain and simultaneously incubated in the climate chamber to control for the
2 time gap for growth. This is necessary because penetration can only be recorded by
3 observing growth of *Salmonella* on the agar.

4 **Inoculation and storage**

5 For each experiment, ca 340 whole eggs and 100 agar-filled eggs were exposed to
6 streptomycin resistant SE MB1409 by dipping for 1 min in the immersion solution.
7 Both the eggs and the immersion solution were at 20°C. All eggs remained at
8 ambient conditions until dry and were then placed in a climate chamber (Termaks
9 KBP 6395 F, Solheimsviken, Norway) for up to 20 d.

10 **Determination of shell contamination**

11 On the day of inoculation the *Salmonella* shell contamination of 20 whole eggs was
12 analysed to determine the inoculation dose. Also, 3, 8, 15 and 20 d later, ca 80 whole
13 eggs were taken at random from the climate chamber and their shell contamination
14 was examined. At the end of storage, the *Salmonella* shell contamination of all agar-
15 filled eggs was also determined.

16 Each egg was gently washed in 10 ml PBS (Oxoid) in a sterile plastic bag by
17 rubbing the surface of the eggs, through the plastic bag, for 1 min. Enumeration of
18 *Salmonella* in the PBS solution was done by plating out 1 ml on XLD agar (Oxoid)
19 and incubation for 24 h at 37°C. In case of high counts, serial decimal dilutions of
20 the PBS solution (stored refrigerated overnight) in ¼-strength Ringer solution
21 (Oxoid) were made and plated out on XLD agar (Oxoid). In case of no counts, the
22 PBS solution was enriched with BPW (Oxoid) at a ratio of 1:3 (v:v), incubated
23 overnight at 37°C and a loop-ful of this solution was streaked on to an XLD (Oxoid)
24 plate and incubated for 24 h at 37°C. When there were no colonies in 1 ml PBS
25 solution, but there was a positive plate after enrichment of this PBS solution, a

1 counts of 5 cfu/eggshell (or 10 ml PBS solution) was given. When there were no
2 colonies after enrichment, a count of 1 cfu/eggshell was given to allow log
3 transformation.

4 **Statistical analysis**

5 One-way ANOVA was used to study the effect of the storage conditions on
6 *Salmonella* shell contamination using Statistica 6.1 (Statsoft Inc., Tulsa, USA). Non-
7 central interval estimation in the power analysis module was used to calculate the
8 confidence intervals (CI) at 95% confidence of the percentage of penetrated
9 eggshells at the various storage conditions.

10 **RESULTS**

11 **Survival of *Salmonella* on the eggshell**

12 The survival of SE MB1409, inoculated on the shell of whole eggs, was assessed
13 during storage at various environmental conditions. The mean initial contamination
14 dose was 2.57 ± 0.80 log cells per egg.

15 The percentage of shells positive for *Salmonella* by enrichment is depicted in
16 Table 1. With increasing storage time, the percentage of contaminated shells
17 decreased and was most pronounced at 75% RH. While more than 90% of shells
18 remained contaminated after 3 d of storage at 60% and 45% RH, at 75% RH fewer
19 than 43% remained contaminated. Particularly when combined with the higher
20 temperature of 25°C, contaminated shells became scarce. At 45% RH, survival at
21 15°C was higher than at 25°C. When the eggs were stored at 20°C-60% RH, survival
22 was comparable to storage at 25°C-45% RH.

23 The survival experiments showed considerable variability. Average
24 *Salmonella* counts on the shell declined over time (Figure. 1). However, following

1 inoculation, bacteria either died off partly or completely (shells negative by
2 enrichment, Table 1) or started to grow up to a maximum value of 7 log cfu/shell.
3 Growth was observed in a similar proportion of eggs irrespective of the storage
4 conditions tested (Table 1). Thus, although a large percentage (71.3%) of eggshells
5 were positive after enrichment at 15°C-45% RH; growth was observed on only 6.3%
6 of the eggshells, that is, on 8.8% of the eggshells that were positive after enrichment.
7 In contrast, at 25°C-75% RH, only 8.8% of eggs were positive after enrichment, but
8 on 86% of the positive shells growth was observed.

9 **Influence of storage conditions on eggshell penetration**

10 Eggshell penetration results of agar-filled eggs stored at various environmental
11 conditions are shown in Figure. 2. Storage temperature affected the initial rate of
12 penetration by SE MB1409. At higher temperatures red spots on the agar within the
13 eggs appeared more quickly; at 25°C, shell penetration was observed most frequently
14 at day 2; at 20°C and 15°C, most shells were penetrated at day 3 and day 6,
15 respectively. At day 7, day 8 and day 13, more than 95% of shell penetration was
16 observed at 25°C, 20°C and 15°C, respectively. Relative humidity of storage was not
17 a significant factor. At the end of storage, on average 44.7% of eggs were
18 contaminated. Although the percentage of penetrated eggs ranged from 35.4% (CI
19 25.9-45.8%) to 51.5% (CI 41.3-61.7%) for the various storage conditions, the
20 differences were not significant.

21 **Influence of shell contamination on eggshell penetration**

22 Using the agar approach (Figure 3a) shell contamination at 20 d of storage was
23 higher ($P<0.0001$) for the shells that were penetrated compared to those that were not
24 penetrated. The higher the contamination at the end of storage, the higher the
25 probability that the eggshell will be penetrated (Table 2). Shell contaminations

1 exceeding 4 log cfu yielded more than a 90% probability for an internally-
2 contaminated egg. Shells contaminated with a value of less than 10 cfu had a 50%
3 probability of becoming internally contaminated.

4 In Figure 3b, shell contamination of agar-filled eggs at 20 d of storage for the
5 various storage conditions is depicted. The median value for each storage condition
6 was 1 cfu/shell. *Salmonella* was not recovered from 59.3% (CI 48.9-69.3%), 63.0%
7 (CI 52.8-72.4%), 67.4% (CI 57.0-76.7%), 52.5% (CI 42.2-62.7%) and 70.0% (CI
8 60.0-78.8%) of the shells after 20 d of storage at 15°C-45% RH, 15°C-75% RH,
9 20°C-60% RH, 25°C-45% RH and 25°C-75% RH, respectively. No significant
10 difference was found in mean value on the shell for the various storage conditions
11 ($P=0.094$).

12 *Salmonella* counts on the shell of whole (Figure 1)
13 and agar-filled eggs (Fig. 3) combining all storage conditions were compared. The
14 percentage of shells where *Salmonella* could no longer be recovered from was 68.2%
15 (CI 63.3-72.8%) for the whole eggs and 62.5% (CI 58.0-66.8%) for the agar-filled
16 eggs. Statistical differences were not found.

17 DISCUSSION

18 Previous studies by our group (De Reu *et al.*, in press; Messens *et al.*, 2005a)
19 indicated that *Salmonella* shell contamination at the end of storage correlated
20 strongly with eggshell penetration. The present study was set up to examine the
21 relationship between egg storage conditions (with corresponding survival/growth of
22 SE on eggshells) and the penetration of SE through the eggshell. Agar-filled eggs
23 were used to study the actual penetration through the eggshell and its membranes,
24 without interference of the albumen. In other studies, using whole eggs, no
25 differentiation between penetration and survival/growth in albumen was made; for

1 example Braun *et al.* (1999) have shown penetration to be enhanced at higher
2 temperatures although survival on the eggshell was poorer. De Reu *et al.* (in press)
3 compared eggshell penetration (agar approach) and whole egg contamination (intact
4 egg approach) for SE upon storage at 20°C and 60% RH: 43% of the agar-filled eggs
5 were penetrated and 33% of the whole eggs. In that study, the behaviour of various
6 bacterial species on the eggshell was compared. This showed that although some
7 strains were able to penetrate, they could not pass the albumen barrier.

8 Prevention of eggshell penetration or cross-contamination from eggshells
9 contaminated with *Salmonella* can be achieved by decontaminating the eggshell.
10 Several methods can be applied: washing eggs (Hutchison *et al.*, 2004), ionising
11 radiation (Farkas, 1998; Davies and Breslin, 2003b), UV radiation (Rodriguez-Romo
12 and Yousef, 2005) and ozone (Davies and Breslin, 2003b; Rodriguez-Romo and
13 Yousef, 2005), and ultrasonication (Cabeza *et al.*, 2005). Currently, these methods
14 are not allowed in the European Union for class A table eggs.

15 When eggshells are contaminated with *Salmonella*, our study indicates that
16 *Salmonella* remained viable on the shell during storage more often when the
17 temperature was lowered from 25°C to 15°C and especially at a low RH. Enhanced
18 survival at lower temperatures has been previously reported (Rizk *et al.*, 1966;
19 Baker, 1990; Braun *et al.*, 1999; Radkowski, 2002). The RH during storage also
20 plays a crucial role: at 75% RH, bacteria died off rapidly even at low temperature.
21 Simmons *et al.* (1970) have shown that Salmonellae on shell surfaces survive better
22 as either the temperature or RH is lowered farther from the optimum for growth.
23 Bacteria survived longer at 10°C compared to 15°C and 23°C, especially when the
24 RH was lowered from 97% to 75%. We have shown that this is still valid when
25 lowering the RH to 45%. Salmonellae probably survive longer due to the slower

1 metabolism induced by the disadvantageous conditions on the dry eggshell surface
2 (Radkowski, 2002). Extrapolating our results to practical situations, *Salmonella*
3 organisms on the eggshell will die off rapidly in the laying unit as a result of the high
4 RH. In the egg-packing plant, survival will be higher due to the lower temperature
5 and RH. Davies and Breslin (2003a) concluded that contamination in egg-packing
6 plants may be a significant contributory factor to external contamination of shell
7 eggs. In the store during summer, survival will be intermediate; during winter,
8 survival will be low. As observed previously (Rizk *et al.*, 1966), average *Salmonella*
9 counts declined over time. On most shells numbers decreased and *Salmonella* could
10 no longer be recovered from the shell, even with enrichment. Remarkably however
11 on a limited proportion of shells growth of *Salmonella* took place. Although survival
12 rates on the shell differed greatly between the various storage conditions, the
13 proportion of eggshells where growth occurred was not statistically different. In
14 total, ca 10% of the eggshells carried > 4 log SE and some shells were contaminated
15 with as many as > 7 log units per shell. Thus, when eggs reach the consumer, the
16 shell of a relatively small proportion of eggs might be contaminated heavily and give
17 rise to cross-contamination in the kitchen.

18 Although temperature enhanced the rate of appearance of red spots on the agar
19 within the eggs, this does not prove faster initial penetration, because faster growth
20 of SE at higher temperatures was also observed on a control agar plate. On the
21 control plate, pronounced growth was obvious after 1.5 d, 2 d and 3 d at respectively
22 25°C, 20°C and 15°C. At the end of storage, a not significantly different proportion
23 of eggs was contaminated, indicating that eggshells are prone to become penetrated
24 at the same frequency at all conditions studied. Elevated temperatures will however

1 enhance the subsequent multiplication of *Salmonella* in the egg contents as shown in
2 other studies (Schoeni *et al.*, 1995; Fleischman *et al.*, 2003).

3 In conclusion, our study indicates that Salmonellae on shell surfaces survive
4 better as either the temperature (range 10°C to 23°C) or RH (range 45%-75%) is
5 lowered. Growth occurred on a limited proportion of shells resulting in high numbers
6 of SE at the end of storage. Overall, the percentage of penetrated eggshells was not
7 affected by the storage conditions, but strongly affected by the shell contamination at
8 the end of storage. Decontamination of the eggshell is required to prevent eggshell
9 penetration.

10 ACKNOWLEDGEMENTS

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13 Department of Pathology, Bacteriology and Avian Diseases (Ghent University) is
14 acknowledged for the *Salmonella* antibodies testing.

Table 1. *Percentage of eggshells positive for Salmonella by enrichment and percentage of eggshells having counts > 2.57 log cfu/shell (i.e. mean inoculation count; in italics) as a function of storage time at various environmental conditions¹*

Storage time (d)	Temperature and relative humidity of egg storage				
	15°C-45% RH	15°C-75% RH	20°C-60% RH	25°C-45% RH	25°C-75% RH
3	91.3 (82.8-96.4) ^C <i>3.8 (0.8-10.6)</i>	42.5 (31.5-54.1) ^B <i>10.0 (4.4-18.8)</i>	93.7 (85.9-97.9) ^C <i>1.3 (0-6.9)</i>	91.3 (82.8-96.4) ^C <i>6.3 (2.1-14.0)</i>	14.2 (7.4-24.1) ^A <i>10.4 (4.6-19.5)</i>
8	88.8 (79.7-94.7) ^D <i>6.3 (2.1-14.0)</i>	27.9 (18.4-39.1) ^B <i>11.4 (5.3-20.5)</i>	76.3 (65.2-85.3) ^{CD} <i>6.6 (2.1-14.7)</i>	66.7 (55.1-76.9) ^C <i>6.4 (2.1-14.3)</i>	15.4 (8.2-25.3) ^A <i>11.5 (5.4-20.8)</i>
15	87.5 (78.2-93.8) ^D <i>3.8 (0.8-10.6)^{AB}</i>	20.0 (11.9-30.4) ^{AB} <i>15.0 (8.0-24.7)^B</i>	46.7 (35.1-58.6) ^C <i>0 (0-3.9)^A</i>	29.5 (19.7-40.9) ^{BC} <i>3.9 (0.8-10.8)^{AB}</i>	10.1 (4.5-19.0) ^A <i>8.9 (3.6-17.4)^{AB}</i>
20	71.3 (60.1-80.8) ^C <i>6.3 (2.1-14.0)</i>	25.3 (16.2-36.4) ^{AB} <i>15.2 (8.1-25.0)</i>	32.4 (22.0-44.3) ^B <i>2.7 (0.3-9.4)</i>	20.0 (11.9-30.4) ^{AB} <i>3.8 (0.8-10.6)</i>	8.8 (3.6-17.2) ^A <i>7.5 (8.8-15.6)</i>

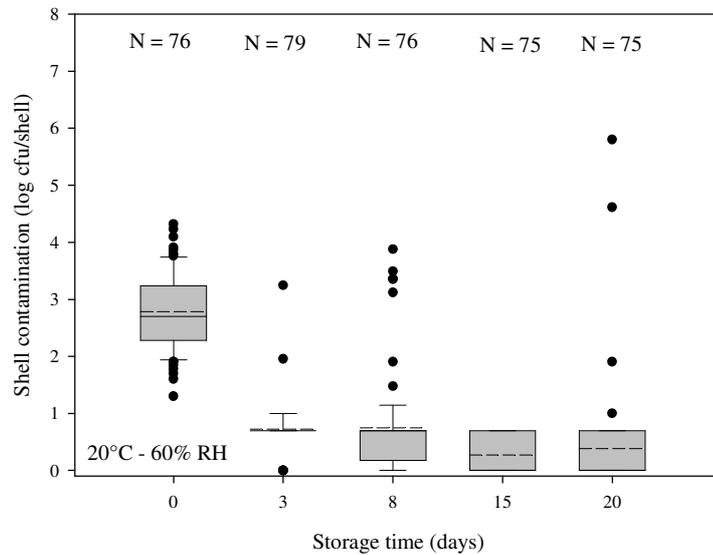
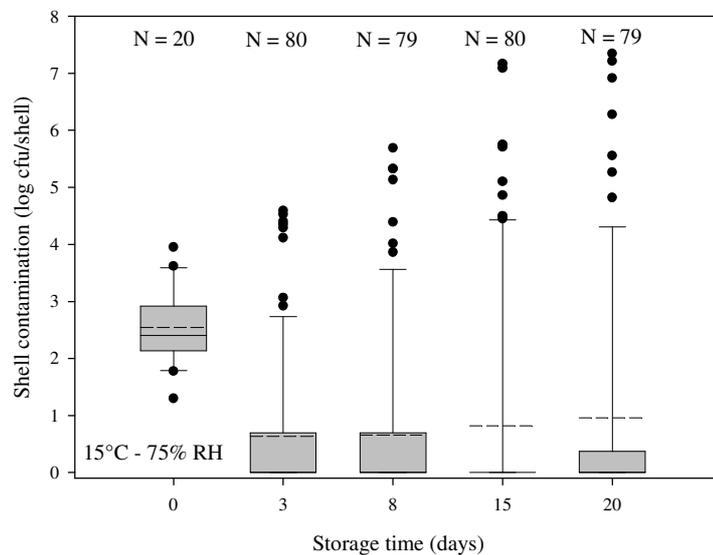
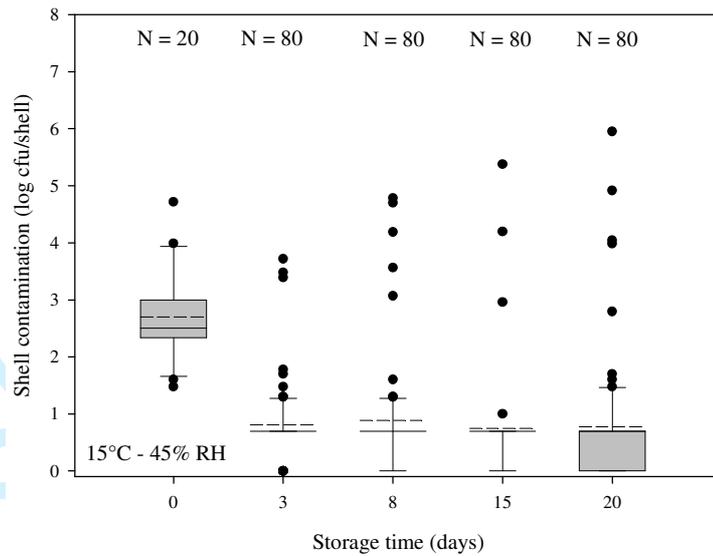
¹74-80 eggs sampled. Means and 95% confidence interval (between brackets) are given. Values in the same row without common superscripts are significantly different.

Table 2. *Influence of Salmonella shell contamination at the end of storage on the percentage of penetrated eggshells*

<i>Salmonella</i> shell contamination	Penetrated eggshells (%)	N
Not contaminated	19	453
< 1 log cfu/shell	51	113
1-2 log cfu/shell	68	41
2-3 log cfu/shell	86	36
3-4 log cfu/shell	90	62
4-5 log cfu/shell	95	39
5-6 log cfu/shell	94	18
6-7 log cfu/shell	92	13
≥ 7 log cfu/shell	100	5

N = number of eggs sampled

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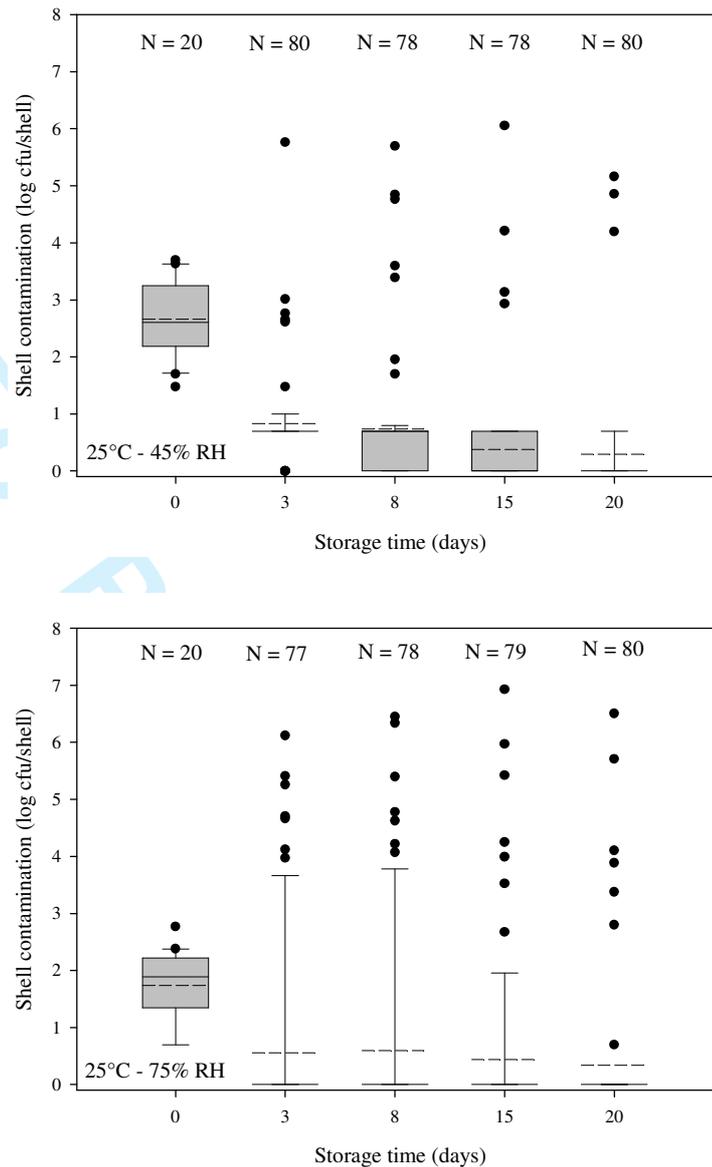


Figure 1. *Salmonella* shell contamination on whole eggs as a function of storage time for various environmental conditions. The number of eggs sampled (N) is shown. The solid line within the box marks the median, while the dashed line marks the mean. The boundaries of the box represent the 25th and 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. The outliers, all data points that lie outside the 10th and 90th percentiles, are shown as symbols.

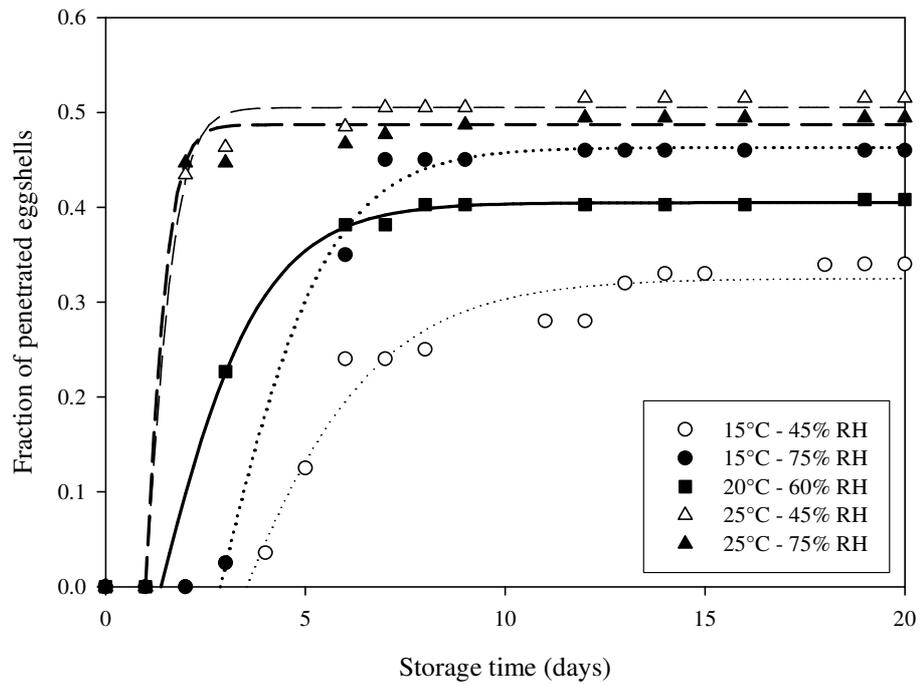


Figure 2. Cumulative fraction of eggshells penetrated by Salmonella as a function of storage time at various storage conditions.

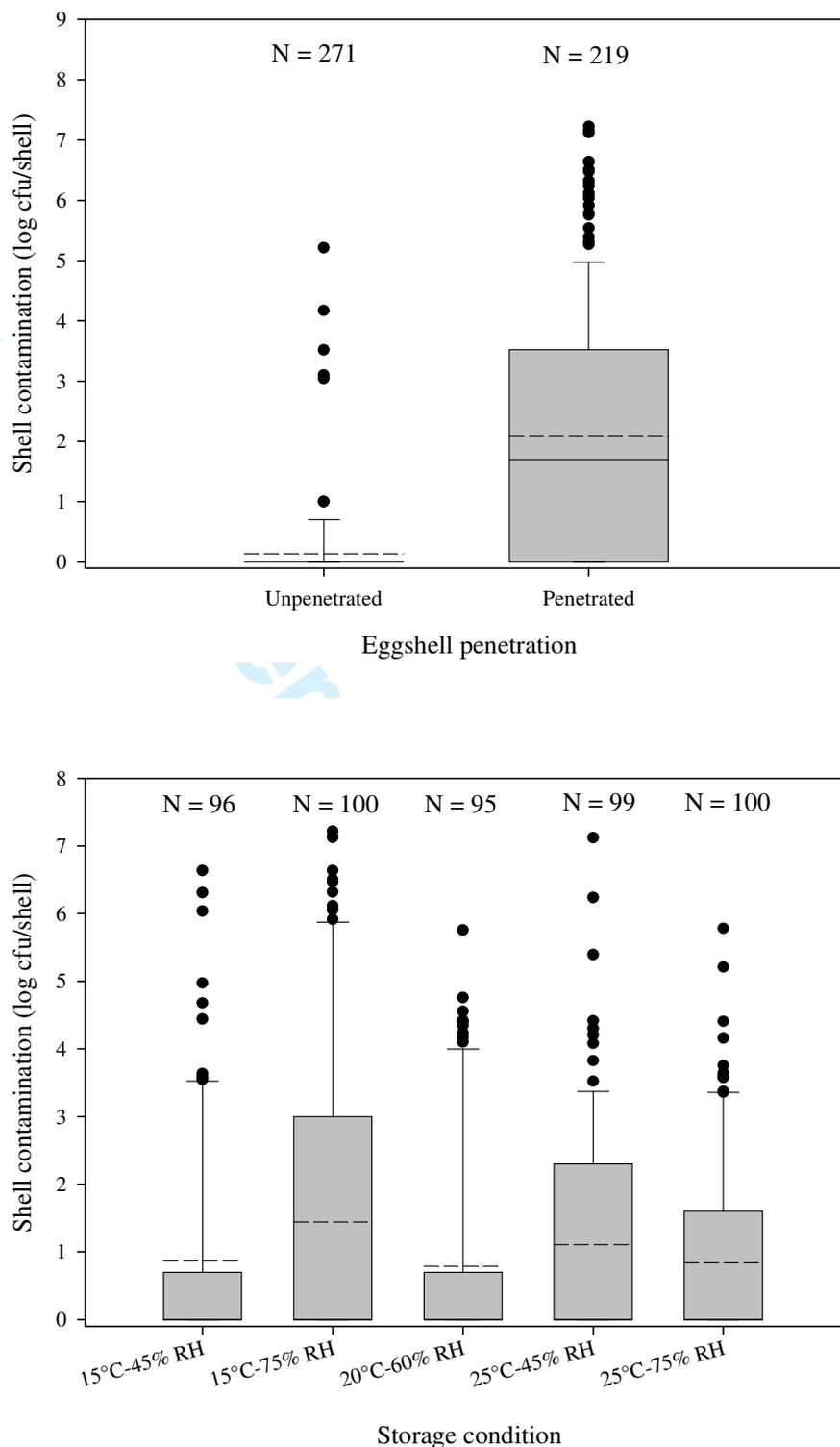


Figure 3. *Salmonella* shell contamination of agar-filled eggs at 20 d of storage for (a) unpenetrated versus penetrated eggshells and for (b) various storage conditions. Box plot as in Figure 1.

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