ANNEX B

Distribution of *Salmonella* Prevalence in Hens and Eggs

This annex addresses the first stage of a farm-to-fork quantitative risk assessment designed to model the human-health risk attributable to *Salmonella*-contaminated eggs. It provides data analysis and support for modeling the percentages of *Salmonella*-positive eggs produced by *S.* Enteritidis (SE)-infected flocks (defined as hens that could become infected due to the presence of SE in the environment, as measured by the National Animal Health Monitoring System (NAHMS)[1]) by a vertical or through shell route of infection. These percentages may depend upon several biological and husbandry factors; therefore a probability designed national survey of flocks would be needed to estimate the distribution of the percentages of infected eggs. However, as no such survey has been conducted, it is necessary to model the distribution in an indirect fashion by considering various data sources.

To model the percentages of *Salmonella*-infected eggs U.S., the following process was used. The percentage of flocks with *Salmonella* is modeled first. Then the percentage of *Salmonella*-infected hens in an infected flock is modeled. Next the percentage of eggs contaminated with SE by transovarian infection, or *Salmonella* on the shell is modeled. The product of these three percentages provides an initial estimate of the likelihood that an egg is *Salmonella*-infected. To account for the change in likelihood of infection due to their time of molting relative to egg laying, the weekly infection rate per egg is then multiplied by a different molting factor each week post-molt for 10 weeks. The location of the infection within the egg is also considered to

allow for subsequent differential SE growth rates based on the location of the initial infection. For the case of eggshell contamination by *Salmonella* spp., the percentage of eggs that become *Salmonella* spp. infected by through shell penetration is modeled. Due to the significant uncertainty in the science, false negative rates of sampling methodology, and the natural variability in the egg production system many of these values have been estimated using probability distributions.

The annex provides estimates of values of parameters that are identified in the models that are used, and their associated uncertainties. These parameter values will ultimately be used in the Exposure Assessment/Risk Characterization along with outcomes of other annexes to determine the risk of *Salmonella* egg contamination to the consumer.

This annex provides data analysis and support for modeling:

1) The prevalence of SE and *Salmonella* spp.-infected flocks in the U.S.
2) The distribution of the percentage of SE and *Salmonella* spp.-infected individual hens within a flock.
3) The prevalence, near the time of lay, of SE-positive eggs produced by SE-infected hens in SE-infected flocks by a transovarian infection.
4) The prevalence of *Salmonella* spp.-positive eggs produced by *Salmonella* spp.-infected hens in *Salmonella* spp.-infected flocks by through shell penetration infection.
5) A weekly molting factor to capture the likelihood of contaminated eggs being laid by SE-infected molted hens for 10 weeks.
6) The percentages of infection sites within an egg:
    a. In the yolk (*Ey*)
    b. On the vitelline membrane (*Ev*)
    c. Near the yolk but in the albumen (*Eac*)
    d. Farther away from the yolk but in the albumen (*Eaf*)
    e. In the inner shell membranes (*Es*)
    f. On the outer egg shell

The primary outputs of this annex are probability distributions to estimate the likelihood that an egg produced by any one of two routes of transmission under molted or non-molted status is SE-infected. That is, one distribution for each of the three conditions below:

1) SE-infected molted flocks via transovarian infection.
2) SE-infected non-molted flocks via transovarian infection.
3) SE-infected non-molted flocks via shell penetration.

Most of this annex is devoted to the estimation of the parameters and relationships essential to the development of these distributions. This annex provides the procedures and the rationale used in estimating the above outputs.

THE DATA

The data in this annex were acquired by web-based electronic searches using pertinent search terms. References from relevant articles were assessed to acquire additional journal and book publications. Raw and unpublished data were obtained by direct correspondence with the investigator and expert opinion was utilized. Data were analyzed by a weight of evidence approach. That is, scientific publications were analyzed and interpretations made based on a preponderance of the evidence.

The estimate of the percentage of SE-infected flocks nationally was based on SE environmental sampling data from NAHMS.[1] In addition, the USDA National Agricultural Statistics Services (NASS)[2] and Pennsylvania SE Pilot Project[3] data were used as weights to account for regional SE prevalence differences and environmental false-negative sampling, respectively.

From the population of infected flocks, the distribution of the within-flock percentage of infected hens that would be laying infected eggs was determined. Though there was no direct data known for estimating this distribution, this assessment used a summary of the results from a 1991 and 1995 spent hen survey.[4] (Spent hens are those that have passed efficient commercial egg production limits and thus are sent to slaughter.) Therefore, the percentage of infected hens in spent hen flocks is used in this assessment as a proxy of the percentage of hens in the laying hen population that are infected and potentially producing infected eggs.

To determine the prevalence of SE-positive eggs produced by SE-infected hens in SE-infected flocks by a transovarian infection the within-flock prevalence was multiplied by the percentage of SE-infected eggs. This latter percentage was estimated from eggs collected from experimentally SE-inoculated hens over an 8-week period.[5] Additionally, as molting is known to increase the percent of SE-infected eggs by infected hens, data from SE-positive eggs collected after molting practices were used to determine weekly molting factors for 10 weeks.[5]

The percentage of *Salmonella*-infected eggs produced by through-shell penetration was modeled in a similar fashion for that of transovarian infection. Spent hen surveys were used to determine the percentage of *Salmonella* spp. infected flocks and to estimate the within-flock prevalence of *Salmonella* spp. An experimentally infected hen study was used to determine the percentage of surface infected eggs and the percentage of through shell infections was determined using data from Schoeni et al.[6]

Taken together, these analyses provide the number of transovarian or shell penetrated infected eggs produced by a molted or a non-molted infected flock. These outputs then are used as inputs to the exposure assessment/risk characterization.

From the population of infected eggs, we sought to determine the distribution of infection sites from either through shell penetration or transovarian transmission. By these mechanisms SE can be deposited on the eggshell surface and within several different egg compartments (*Ey, Ev, Eac, Eaf* and *Es*), respectively. Identification of these percentages relative to one another is important as the site of infection will influence the subsequent growth rate within the egg. The growth of SE within the egg is a principle risk factor for consumers. These percentages discussed below were estimated from published studies of experimentally infected hens. The percentages of each of these six infection sites are estimated by a distribution to account for the variability and uncertainty in the estimate.

The above procedures depend upon many assumptions. To evaluate these assumptions and the procedures used, an extensive review of the published literature was prepared to investigate

the factors that would influence numbers of infections and the levels of them. This includes a discussion regarding the usefulness of data obtained from experimentally inoculated hens compared with naturally infected hen studies (Attachment B1). Therefore, this annex provides an in-depth analysis of many of these features listed above to better understand the molecular mechanisms that result in much of the variability observed for SE infection in hens and how this relates to the frequency of *Salmonella* within infected eggs. The data and information presented in this annex were used to formulate assumptions and construct models throughout the other parts of this risk assessment. A summary of these models and the assumptions to be used in modeling is given at the end of the section.

Proportion of SE Positive Flocks

Estimation of the number of SE positive eggs in the U.S. begins with an estimate of the proportion of flocks in the U.S. that are SE positive. A SE-infected flock is defined has having one SE positive sample and assumed to have at least one SE positive hen. The presence of SE within a flock will vary in the U.S. The reasons for variation among flocks are likely due in part to husbandry issues such as rodent index, production house temperature and humidity, ventilation, stocking density, caging and feeding/watering systems. These factors could create a more habitably and transmission-favorable environment for SE to exist. These issues will not be discussed in this risk assessment; however, they serve to demonstrate variability of SE among flocks is expected. Below, this risk assessment provided the data support and reasoning used to estimate the national percentage of SE positive eggs.

NAHMS Layers '99 Survey

In 1999, NAHMS conducted a survey to estimate the prevalence of SE in layer flocks from 15 selected U.S. states.[1] Environmental sampling was conducted from May 3rd through Oct. 22, 1999 in 200 layer houses. These 200 houses resided in 15 states and represented over 82% of the 1997 laying hens in the U.S. One house per farm was typically chosen at random for environmental sampling. At larger farms multiple houses were sampled. Five manure samples, five egg belt samples, five elevator samples, and two walkway samples were gathered for each house (two swabs per sample). These samples were then shipped on ice for culturing to the Agricultural Research Service in Athens, GA. From these data, this survey estimated approximately 7.1% of the flocks in the 15 selected U.S. states were positive for SE with a standard error of 3.6%. This large standard error reflects the limitations of small sample size. A regional analysis of the sample results is presented in Table B1. Sample proportions and their estimated standard errors are presented for four regions.

Adjusting for regional differences

The NAHMS information was reported by region a shown in the table. To account for the differences in the total number of flocks housed by these different regions, regardless of the presence of SE, additional data was used. A 1999 USDA-National Agricultural Statistics Services (NASS) survey[2] was used to identify the percentage of total U.S. flocks by region,

regardless of SE status. Using these percentages as weights, the NAHMS national estimate of SE-positive flocks[1] was adjusted to be 9.6% with a standard error of 5.2%. The addition of the NASS data therefore allowed for a more accurate national estimate of SE-positive flock prevalence.

Adjusting for false-negative test results

TABLE B1 NAHMS RESULTS FROM 1999 NATIONAL SURVEY USING ENVIRONMENTAL DRAG SWABS.

Region	% Flocks SE-positive	% U.S. Flocks In Region
Great Lakes	17.2 (13.7)	35%
Southeast	0.0 (--)	15%
Central	9.0 (7.2)	28%
West	4.4 (2.5)	22%
Total	9.6 (5.2)	N/A

Environmental sampling can underestimate the percentage of positive flocks due to false negative results and low levels of SE being shed by infected birds. An adjustment for false negative results was made as follows using data from a field trial conducted by Schlosser et al.[3] For environmental swab sampling, about 48% of infected flocks would be positive on a single test. In that study, a single flock test usually consisted of collecting separate swab samples from each manure bank (typically 6 samples per flock), each egg belt (typically 6 per flock), and other surfaces in the poultry house (typically 4 samples from walkways or walls). In the field trial, 12 flocks' environments were sampled weekly for 12 consecutive weeks. Eight of the flocks had at least one positive test result during the 12 weeks of sampling. Among these 8 flocks there were 46 positive results from 95 environmental collections; apparently one test result was missing. Assuming these 8 flocks were positive for all 12 weeks, the above result implies an approximate 50% false negative rate. Consequently, the proportion of positive flocks in the NAHMS study is multiplied by a factor of approximately 2 (95/46) to adjust for underestimation of infected flocks based on false negative results.[a]

Flock prevalence estimate

The proportion of infected flocks in the U.S. was estimate to be 7.1% with a standard error of 3.6% based on the NAHMS survey results. This proportion was adjusted for regional differences, 9.6±5.2%, and then multiplied by a factor of two to account for false negative test results. Consequently, the prevalence of SE-infected flocks is assumed to be 19.2% with a standard error of 10.4%.

[a] To apply the false negative rate of Schlosser et al.[3] to the data from the NAHMS survey,[1] testing procedures were evaluated for both studies. The sampling and culturing procedures employed by Schlosser et al. are somewhat comparable to that used in the NAHMS survey. Therefore, the Schlosser et al. false negative rate that was applied to the regionally adjusted NAHMS survey estimation of SE-infected flocks.

SE-infected Birds in an SE-positive Flock

Given the proportion of infected flocks as estimated above the next task was to estimate the proportion of birds in an infected flock that were SE positive. The number of individually infected hens within an infected flock is likely to differ among flocks by region and season. This variation could be due to differing rates of SE transmission among birds within an infected flock. Factors affecting this are likely to be conditional on hen and SE strain genotype variability. Environmental and husbandry factors such as rodent index, production house temperature and humidity, ventilation, stocking density, caging and feeding/watering systems will also alter transmission rates. Additionally, mitigation strategies such as vaccination and competitive exclusion have been used to lower the likelihood of intestinal colonization by *Salmonella* spp. and therefore lower the shedding of these bacteria. Due to the many factors that could affect the proportion of infected hens within a flock, variability is expected among flocks. The procedures used to estimate this proportion are given below.

Prevalence of SE in spent hens

To estimate the proportion of birds in an infected flock that were SE positive, two national studies utilizing spent hens[2] at the time of slaughter in 1991[7] and 1995[8] were used. These studies are the only national surveys we know of that attempt to quantify SE within-flock prevalence.

This risk assessment recognizes that the use spent hens to estimate the SE within-flock prevalence of younger laying hens is uncertain. Ebel et al.[7] said, "Because the bird samples in this [the 1991 spent hen] survey were at the end of production, it is uncertain whether these results represent recent colonizations acquired during transport to slaughter or chronic colonizations acquired earlier in production, or whether over time a house of birds will accumulate a certain prevalence of colonization." Therefore, even though the spent hen survey data is applicable to older hens at the time of slaughter, the usefulness of spent hen survey data to predict the likelihood of commercial within-flock SE prevalence is unclear.

For instance, variation in within-flock prevalence is expected due to the dynamic nature of SE; however, the spent hen surveys indicted that most SE positive flocks have relatively few SE positive hens. Seventy-seven flocks had only one positive sample test among the average of 58 tests per flock and 247 SE-positive flocks. This suggests 31% (77/247) of the infected flocks had low within-flock prevalence. The highest number of positive tests was 44 for 1 flock out of 247 SE-positive flocks. This suggests 0.4% (44/247) of SE positive flocks have high within-flock prevalence.[7,8] These data suggest SE prevalence within an SE-positive flock could be low for most SE-positive flocks. However, the number of tests per flock is uncertain. If this number is low due to missing test samples, this would imply greater within-flock prevalence. Therefore, depending upon the number of samples actually tested for each flock, the estimate of the percentage of infected hens could be higher. In an effort to understand these data several factors that could influence the estimated within-flock prevalence of hens are discussed in the following paragraphs.

Age of spent hens

Spent hens are more likely to be older than hens used to produce eggs. Therefore the hens used in the 1991 and 1995 spent hen surveys will be birds about 2 years of age. The age of spent hens suggest they will be physiologically different than hens of laying age. This physiological difference might affect the within-flocks prevalence of SE.

Hens can consistently produce eggs at a normal rate for about 45 weeks. This is followed by a decline in egg production that varies with hen breed. Therefore, producers will molt their hens once at 45 weeks of age, a procedure that rejuvenates the egg-laying rate. Post-molt, hens will often be kept for egg production until they are 100 weeks old; some producers molt their hens a second time at 100 weeks. This depends on the current market. Hens are occasionally kept for 120 weeks. Consequently, spent hens might be between 1 and 2.5 years olds. Most spent hens will be about 2 years of age because the majority of production houses molt their hens once.

Prevalence of *Salmonella* spp. in Laying Hens

The text suggests the prevalence of *Salmonella* spp. in spent hens is very high, implying non-SE *Salmonella* might be more competitive in spent hens. For this to be plausible, the prevalence of *Salmonella* spp. in hens of laying age must be lower. However, no U.S. studies were identified investigating the prevalence of *Salmonella* spp. in naturally infected flocks of laying age to determine the baseline frequency of *Salmonella* spp. in these hens. Below, some studies are considered to elucidate the prevalence of *Salmonella* spp. in laying hens.

An article identifying *Salmonella* spp. from the wash of chicken carcasses and raw ground chicken was used to determine a baseline for the presence of *Salmonella* spp. in broiler chickens.[9] These authors reported that chickens can harbor many different *Salmonella* serotypes, and as an upper bound, they found 26.2 and 30.0 % of chicken wash and raw ground chicken contaminated with *S.* Heidelberg (among other *Salmonella* serotypes), respectively. However these percentages are difficult to compare directly with the spent hen surveys as these birds are broilers and have gone through levels of processing that might environmentally contaminate some samples.

Two Canadian studies identified layer flocks as most often contaminated with *S.* Heidelberg at frequencies of 20 and 10%.[10,11] These two studies are difficult to compare directly to the spent hen surveys as differences in Canadian production might affect the epidemiology of *Salmonella*. Also, these studies assayed hen fecal droppings and other environmental sampling that might underestimate the prevalence of *Salmonella* compared with cecal sampling.

These data suggest the baseline of commercial hens infected with *Salmonella* is relatively low compared with those in spent hen surveys. This difference in prevalence is likely due to the increased susceptibility of older[12] and molted hens[13] to infection.

Susceptibility to SE and competing Salmonella *spp.*

To explain why so many infected flocks have so few SE-infected hens as determined by the 1991[7] and 1995[8] spent hen surveys, the effect of age on colonization by SE was analyzed. It appears older hens have weakened immune systems, making them more susceptible to colonization by SE. However, these hens will also be more susceptible to other *Salmonella* spp. SE and other *Salmonella* spp. will likely compete for colonization of certain hen tissues. Therefore, it is difficult to estimate the effect this will have on the prevalence of SE in older hens. This discussion is given below.

The effect of age on colonization of adult hens, i.e., laying egg age, by SE is unclear. It is generally thought that older hens produce weaker immune responses and have extended SE fecal carriage.[12] Studies suggest that the antibody level produced by an immune response of a 62-week

old hen declines more quickly than that of a 37- or 27-week-old hen. It is likely that older hens will be more susceptible to SE infection for longer time periods due to the inability to mount or sustain a 'normal' immune response. Molted hens are more susceptible to SE intestinal colonization and prolonged fecal shedding as compared to non-molted hens.[14,15] These data imply spent hens are more susceptible to SE infection and spent hens might overestimate SE within-flock prevalence. On the other hand spent hens might underestimate prevalence due to the presence of competing *Salmonella* spp.

To investigate the implications of competing *Salmonella* spp., surveys were sought that elucidated the baseline prevalence of *Salmonella* spp. in commercial laying hens. However, no such survey was found (see textbox). Once again, this risk assessment turned toward spent hen data. Spent hen surveys observed a large percentage of flocks are frequently colonized with other *Salmonella* serotypes besides SE: 76.2, 97.4, 86, 98 and 100% respectively as determined by pooled samples of ceca[b] or ovaries.[7,8,16-18] Only one of the studies serotyped non-SE *Salmonella* and found that as an upper bound, 56.5% of the hens were colonized with *S.* Heidelberg.[17] In addition, these surveys identified only 2.4, 1.5, 3.0, 5.1% and 0.16% flocks SE positive. Therefore it appears that spent hens are infrequently colonized with SE, yet can be frequently colonized with non-SE *Salmonella*.

Based on these findings it seems spent hens are likely more susceptible to many *Salmonella* serovars, not only SE. In the presence of competing *Salmonella* strains, this might have the effect of other *Salmonella* serotypes out-competing SE for the same niches within a hen. Therefore, SE might be under represented in spent hens. This could explain why the two spent hen surveys had such low SE within-flock prevalence for spent hens. Taken together, these data suggest the low frequency of within-flock SE prevalence identified in 1991 and 1995 spent hen surveys might under-represent SE prevalence in laying hens.

False negative rate of spent hen survey

Isolation of SE from egg compartments or mixed yolk and albumen is not a reliable process. Albumen can inhibit the growth of SE due to its bacteriostatic nature leading to false negative results. Therefore, studies attempting to measure SE deposited into the albumen or SE from pooled egg contents need to employ various measures to consistently recover SE. Evaluation of the SE recovery methodologies from pooled egg samples used by the 1991 and 1995 spent hen surveys suggests there were false negative results. To most accurately estimate the prevalence of SE it is necessary to adjust for these false negative rates that tend to underestimate prevalence. These factors are reflected in the interpretation of the presented studies to accurately depict SE prevalence in eggs.

[b] Closed intestinal pouches connected to the hen lower intestinal tract.

Waltman et al.[18]

To estimate hen flock prevalence of SE, Waltman et al.[18] pooled ceca from spent hens and incubated the samples in rich medium (TT) for 24 hrs. The culture was then inoculated onto either xylose-lusine-tergitol-4 (XLT-4) plates or brilliant green agar supplemented with 20 µg novobiocin/mL (BGAN). XLT-4 and BGAN plates identified 64% (1536/2418) and 72% (1740/2418), respectively, of the total *Salmonella* positive cecal samples (82% (1993/2418) together). If these procedures were negative, an extended incubation in TT broth was then performed and streaked onto the two plate types. This latter method identified 425 more positive samples. XLT-4 medium was designed for recovery of Group D *Salmonella* (includes SE) where BGAN can identify a broader range of *Salmonella* serotypes. Therefore it is not unexpected that BGAN recovered more isolates. These methods were employed due to the difficulty observed in reliably isolating SE and other *Salmonella* spp.

The 1991 and 1995 spent hen surveys might have underestimated the within-flock prevalence due to false negative recovery rate of SE. This is evidenced by the results of Waltman et al.[18] Of the 6 SE positive samples identified in this study, 3 were isolated on XLT-4 plates, 5 on BGAN plates and 1 by the extended incubation method (see textbox). That is, from the 6 known or at least assumed SE positive samples, the XLT-4 missed 50% of the samples. The levels in the samples were not known, but the levels in some of the missed samples were sufficiently high to be detected by the BGAN. Ebel et al.[7] and Hogue et al.[8] only utilized XLT-4 plating to identify SE within pooled cecal samples and therefore could have missed some SE positive samples. Miller et al.[19] say, "It is suggested that two different types of plating media be inoculated to further reduce the possibility of a false-negative finding that could occur if a particular strain of *Salmonella* were sensitive to an inhibitor used in one of the two media." These data suggest a possibly significant percentage of false negatives could have occurred in these studies due to the methodology. Therefore, within-flock prevalence could be underestimated in the two national 1991 and 1995 spent hen surveys.

Additional evidence to support a false negative sampling rate of the 1991 and 1995 spent hen surveys is given below. Analysis of the ceca, as performed by the 1991 and 1995 spent hen surveys is a good indicator of hen infection by SE,[20-22] as positive cecal culture samples are typically the most frequent when other extra-intestinal tissues are cultured simultaneously. Though it is unclear how hens typically become SE-infected, it is generally thought hens are horizontally contaminated orally through contaminated feed, water or contact exposure and subsequent preening. Even airborne infection has been shown to result in some direct oral contamination;[23] therefore, oral SE exposures may enter the gastrointestinal (GI) tract and the ceca. However, from this initial site of colonization, SE can disseminate systemically and bacterial counts will often fall within the GI tract as the immune system responds to the infection. SE could colonize extra-intestinal tissues and be undetectable in the ceca.

Protais et al.[22] showed at 28 days post-inoculation one experimentally inoculated hen out of 16 was infected in the liver, spleen and oviduct. It is unknown if these were all in the same hen. None of the hens were SE ceca positive. Using a different hen line these authors demonstrated 9 hens out of 10 were ceca positive yet SE was present in the spleen and the ovary in the one ceca-negative hen. In addition, Keller et al.[20] found in one experiment of 3 that SE was detected in 70.0% of experimentally inoculated hens by culture of a ceca and small intestine pool. Yet organ (heart, spleen, liver and gallbladder) culture of these same hens identified 95.0% of infected hens.

Assuming that the false negative recovery rate is very low, these data suggest that although culture of ceca is a reliable indication of hen infection, a small percentage of hens will be ceca negative yet colonized with SE.[20,22] Furthermore, as stated above, studies that only utilize one

plate type to recover SE might underestimate the frequency of SE due to their methodology. Together, these data suggest the 1991 and 1995 spent hen surveys underestimated the within-flock prevalence of SE due to a false negative rate of unknown magnitude. The application of the false negative rate is discussed in the following section.

Data Analysis of Spent Hen Survey

This risk assessment is using the distribution of the within-flock percentage of infected hens of spent hen flocks as a proxy for the distribution of the within-flock percentage of hens that are infected and potentially producing infected eggs by transovarian infection. The data used are from the 1991[7] and 1995[8] spent hen surveys, summaries of which may be found in FSIS SE risk assessment[4] (Table A3). The uncertainty estimates and difficulties this presents are discussed below. This is followed by discussion of the false negative rate to be applied to the within-flock prevalence.

Estimating within-flock prevalence

Table B2 presents a compilation of the data from the two studies.[7,8] It was reported that 300 hens were sampled from each flock and for each hen, one cecum was examined. Five ceca were pooled and analyses were performed on the pooled samples. Ebel et al.[7] reported that, on the average, 58 samples per lot were analyzed from 406 lots. Since the maximum was 60, this could imply that for some lots, a relatively large percentage of the designated 60 samples were not analyzed. This, create some difficulty in determining the distribution of the percentage of infected hens.

The data in Table B2 show numbers of flocks for which only one or two pooled samples were positive is relatively large. The mode of the distribution of the number of positive samples is 1, suggesting that for most flocks only a relatively small percentage of hens would be actually infected. On the hand, the largest number of positive samples is 44. Let q be the fraction of positive samples within a lot, and h be the false negative rate, then an estimate of the percentage of hens infected, $\hat{p}(q)$, in a flock is given by:

$$\hat{p}(q) = 1 - \left[1 - \frac{q}{1-h}\right]^{1/5} \qquad \text{(B1)}$$

If $h = 0.15$ and $q = 44/58$, corresponding to 58 samples for the flock, then $\hat{p}(q) = 36\%$.

TABLE B2. DATA FROM THE SPENT HEN SURVEYS[7,8] TAKEN FROM THE FSIS SE RISK ASSESSMENT, 1998 REPORT.[4A]

Number of Positive Pooled Samples	Number of Flocks	Estimated Within Flock Percentage of Infected Hens
0	464	0.00
1	77	0.41
2	39	0.82
3	23	1.25
4	18	1.68
5	9	2.12
6	6	2.56
7	8	3.02
8	7	3.48
9	8	3.95
10	4	4.43
11	6	4.92
12	4	5.43
13	4	5.94
14	2	6.46
15	2	7.00
16	6	7.55
17	1	8.11
18	3	8.69
19	3	9.28
21	2	10.51
22	3	11.15
23	1	11.81
24	1	12.49
25	1	13.19
26	2	13.92
27	2	14.67
28	1	15.45
36	1	23.05
39	1	26.89
42	1	31.75
44	1	35.98

[a]Entries are number of positive pooled samples (of 5 ceca), number of lots with this number of positive samples, and an estimate of the within-flock percentage of infected hens, computed, assuming a false negative rate of 15% and 58 samples analyzed per flock.

Let p be the percentage of infected hens within a flock, and assume that the distribution of p is f. The probability of a positive result on a sample, given p and h, is: $q(p) = (1- (1-p)^5)(1-h)$, so that the probability distribution of x positive samples, $b(x|p, n)$, from n tests would be a binomial distribution with parameters n and $q(p)$. Let k_x be the number of flocks with x positive tests, and consider the following measure: $E_x = k_x/(1-b(0|\hat{p}(x, n))$ - the number of flocks with x positive samples divided by an estimate of the probability of at least one positive finding from a flock for which x positive findings were observed. In some rough sense, E_x is an estimate of the number of flocks in the population for which the expected number of positive samples would be x. Thus, for example, E_1 is an estimate of the number of flocks for which it would have been expected 1 positive from n samples.

To "see" the shape of the distribution of the percentage of hens that are infected within infected spent hen flocks, an estimate of the cumulative distribution function, $F(p)$, for $p > 0$, can be obtained by considering the E_x values. For each x there is a corresponding percentage of hens infected in the flock, $p(x)$. The cumulative distribution function, $F(p)$, is estimated as:

$$\hat{F}(p) = \frac{\sum_{x:p(x)LEp} E_x}{\sum E_x} \qquad \text{(B2)}$$

Figure B1 is a plot of the log-log transformation: $t = \ln(-\ln(1-F(p(x)))$ versus $\ln(p(x))$. As is evident from the plot, the data points fall on a straight line, given by: $t = a + b\ln(p)$, where $a = 2.2736$ and $b = 0.5272$. This pattern suggests that a Weibull distribution be used to estimate F.

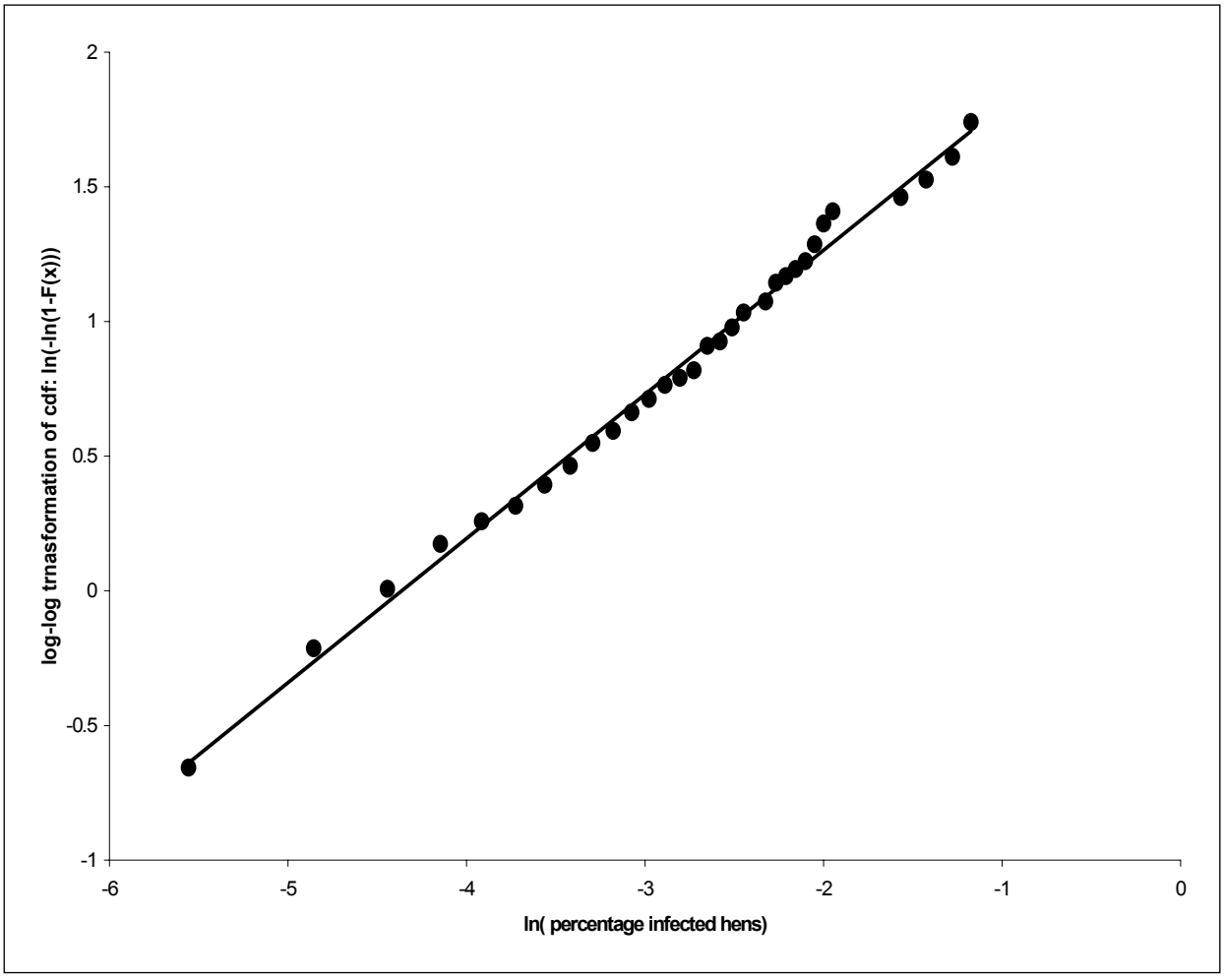

FIGURE B1 PLOT OF LN(-LN(1-$F(P)$)) VERSUS LN(P) WHERE $F(P)$ IS ESTIMATED FROM EQUATION B2.

Taking the inverse transform of t, it is derived that the cumulative distribution function F is approximated as a Weibull distribution: $W(p)$, given by

$$W(p \mid b,c) = 1 - e^{-(p/c)^b} \tag{B3}$$

where $b = 0.5272$ and $c = \exp(-a/b) = 0.01340$.

The above estimates though do not account explicitly for the flocks that have low percentages (< 0.33%) of infected hens and thus would be counted as negative. A more formal estimate, using a maximum likelihood estimation procedure, is made by assuming that the distribution F with density function f is such that for $p>0$, $f(p)$ depends upon parameters of θ, and $f(0)$ is a parameter to be estimated. Thus, based on the above analysis, assume that $f(p)$ is a Weibull distribution with parameters $\theta = (b, c)$. For a given test (a sample of 5 ceca), let $q(p) = [1 - (1-p)^5](1-h)$ be the probability of a positive result. Then, the probability of x positive out of n tests (for a flock) is a binomial distribution with parameters $q(p)$ and n. The likelihood of observing x positive tests, from a total of n tests is

$$\begin{aligned} L\left(x \middle| b, c, f(0)\right) &= f(0)\,(1 - \delta_{>0}) + \\ &\delta_{>0}\,(1 - f(0)) \int_0^1 \binom{n}{x} q(p)^x\,(1 - q(p))^{n-x} f(p \middle| b, c)\,dp \end{aligned} \tag{B4}$$

MLE estimates of the parameters of Equation B4 were determined using Newton-Raphson iteration. The actual estimates were made on transformed values: $\mu = \ln(c)$ and $s = -\ln(b)$, to avoid boundary problems. Convergence was obtained, with a value of $f(0)$ equal to 28.5%. The MLE estimates of the other parameter values of the Weibull distribution were $b = 0.43015$ and $c = 0.005389$. Table B3 gives observed and predicted numbers of samples for given numbers of found positive samples.

TABLE B3 OBSERVED AND PREDICTED NUMBER OF SAMPLES BASED ON MLE ESTIMATES.

No. positive samples per flock	Observed no. flocks	Predicted no. flocks
0	464	464.0
1	77	71.3
2	39	38.6
3	23	25.2
4	18	18.1
5	9	13.7
6	6	10.8
7	8	8.7
8	7	7.2
9	8	6.0
10	4	5.1
11-19	31	25.0
20-52	17	16.9
Totals	711	710.7

The MLE estimates of λ and s, together with standard errors and correlation are given in Table B4.

TABLE B4 MLE ESTIMATES OF PARAMETERS, λ AND S, WHERE B = EXP(-S) AND C = EXP(λ) ARE PARAMETER VALUES FOR WEIBULL DISTRIBUTION: $W(P)$ = 1- EXP(-(P/C)B), WHERE P IS THE FRACTION OF INFECTED HENS IN A SPENT HEN FLOCK.

	λ	s
Estimates	-5.22345	0.84363
Standard Errors	0.36309	0.10775
Correlation		-0.91281

Using these values, the estimate of the 99th percentile is 0.188 with 97.5% upper confidence bound equal to 0.255; the estimated 99.9th percentile is 0.482, with 97.5% upper confidence bound of 0.706.

Estimating the false negative rate

A further concern is possible false negative rates that might occur for a given test. As discussed above,[18] from 6 known SE positive samples, 3 were not detected positive by the methodology used in the spent hen survey. While a 50% false negative rate may be high, such a rate cannot be dismissed, particularly for low level SE-infected flocks. It is possible that the false negative rate would be a function of the percentage of positive test – a higher percentage would, or might imply higher levels of SE, generally, which would imply a lower false negative rate. No information on this is available, and thus, for simplicity, a moderate false negative rate of 15% was assumed in the above analysis.

Proportion of SE-positive Eggs

The purpose of this section is to estimate the percentage of SE-positive egg produced by SE-infected molted and non-molted flocks via transovarian infection, i.e. vertical transmission. These estimates of the numbers of infected shell eggs are used in the Exposure Assessment/Risk Characterization. As discussed above, some flocks are SE-infected and some birds are infected. Infected birds can lay SE-positive eggs. These eggs are infected via transovarian infection. Birds can also lay SE free eggs. These eggs may remain SE free or they can become infected via through shell penetration. To estimate the percentage of eggs laid by transovarian infection, data on the number of eggs produced by eggs experimentally infected over an 8 week period of infection was evaluated. This percentage (q) is multiplied by the percentage of SE-positive hens (p) to estimate the percentage of SE-positive eggs produced by SE-positive non-molted hens.

$$p \times q = pq$$

As molting will increase this percentage, weekly molting factors are developed in this section and applied to the percentage of SE-positive egg per week post inoculation to estimate the percentage of SE-positive eggs produced by SE-positive molted hens. p_1 (% of SE+ hens 1 week post-molt) x q_1 (% of SE+ eggs 1 week post-molt) x m_1 (molting factor 1 week post-molt) = $p_1q_1m_1$ (% SE+ eggs produced by 1 week molted hens by transovarian infection). Data and analysis of how these percentages were estimated is given below.

EGG CONTAMINATION OVERVIEW

SE contaminate the internal contents of eggs by two modes of transmission. SE deposited on the eggshell surface can penetrate the outer shell of a fresh, intact egg thereby gaining access to the internal egg contents. This infection route is known as through shell penetration. Though this certainly posses a risk to the consumer, this mode of transmission is not thought to be the primary method of entry by SE. Alternatively, hen eggs can be vertically contaminated with SE. That is, SE colonization of the ovary and oviduct tissues is thought to infect eggs while the egg is still forming, prior to the formation of the outer shell. There is a correlation between the presence of SE in the reproductive tract and internal egg infection. This route of infection is known as transovarian infection.

Transovarian Infection of Eggs

Direct and indirect evidence exists to support transovarian infection as the primary route of SE egg contamination. Several studies were able to isolate SE from the ovary and oviduct of naturally and experimentally infected hens.[20,21,24-28] The presence of these organisms in the reproductive tract was consistent with the production of SE contaminated eggs in the albumen, the yolk or both. Several studies examining naturally and experimentally infected hens failed to show a strong correlation between SE egg shell contamination and contamination of internal egg contents,[21,29,30] suggesting indirectly transovarian infection.

These data cumulatively suggest transovarian infection of SE to internal egg contents before egg lay is the primary route of infection. Therefore, this risk assessment focused on the percentage of SE-positive eggs produced by transovarian infection to calculate the percentage of SE positive eggs produced by an SE positive flock.

This mechanism of infection and the analyses used to estimate the proportion of SE-positive eggs are described in the paragraphs that follow. The proportion of SE-positive eggs produced by transovarian infection is estimated using data from a study of SE-positive egg production by experimentally inoculated hens over an 8-week period. Then the effect of molting on SE-positive egg production is considered through the development of a factor that can be applied to the proportion of SE-positive eggs. The analysis concludes with a discussion of the possible sites where SE can be deposited within the egg because this is important to future growth of the bacteria.

Mechanisms of transovarian infection

Only infected hens lay infected eggs. Transovarian infection can occur when SE reside in the reproductive tissue of an infected hen. These bacteria can be transferred to the internal compartments of the egg during the egg's formation. Infection of the hen's reproductive system is necessary for transovarian infection. Estimating the percentage of transovarian infected eggs laid by an SE-positive hen is important for subsequent estimates of the frequency of the different types of SE infection in a shell egg. These are important because different types of infections result in different rates of growth of *Salmonella* in the egg and different numbers of bacteria per egg. The number of bacteria in an egg is important in estimating the effectiveness of pasteurization as well as the risk of illness to humans. The purpose of this section is to estimate the proportion of SE-positive eggs that are the result of transovarian infection.

Different experimentally inoculated hen breeds and SE strains have been used to qualify ovary and oviduct SE infection (Table B5). The estimate of the percentage of SE-positive eggs contaminated via transovarian infection begins with evidence of SE colonization of the ovary and oviduct and the level of SE found within these tissues. These data have provided us with a conceptual model of how infection of the ovary and the oviduct lead to internal contamination of eggs: it is believed that colonization of the ovary and its components results in yolk contamination and colonization of various oviduct sections yields differential albumen contamination. However, as indicated below, this conceptual model might be oversimplified.

SE colonization of the ovary and oviduct

A high percentage of the ovaries and the oviducts of hens inoculated with SE are colonized by SE within days of inoculation based on a post-mortem analysis of reproductive tissue.[20,25,27,28] Colonization sustainability, i.e., SE persistence over time, of reproductive tissue was not maintained at the initial prevalence (Table B5-below),[20,21,28] though it is possible SE levels below the culturing detection limit would produce false negatives. The infection appears to be dose-dependent (Table B5).[23,24]

Evidence supporting the idea that hens can be infected with SE below the detection limit is given below. These authors found the reproductive tissue of SE inoculated hens to be free of SE, yet these birds were still capable of producing SE-positive eggs. As infection of the hen reproductive system is thought to lead to internal contamination of eggs, this suggests hens can harbor SE below the level of detection. Gast[24] found no SE-positive reproductive organs with a 4 $\log_{10}$ cfu/hen inoculum, yet 3.8% (3/80) pooled egg contents samples were positive for SE, respectively, supporting the possibility of false negative results. Additionally, Keller et al.[20] found 0% (0/34) reproductive tissue positives at 3 weeks, yet 3.6% (3/84) positive pooled egg contents samples. Therefore, though the magnitude of reproductive tissue infection decreased over time to non-detectable levels, hens still would be capable of producing SE contaminated eggs. These data[20,24] suggest SE ovary or oviduct colonization can be below the level of culturing detection, yet could still contain sufficient numbers of SE to contaminate an egg internally.

TABLE B5 PERCENTAGE OF DOSED BIRDS WITH SE COLONIZATION OF THE OVARY AND OVIDUCT[A]

Publication	Dose	Days post-oral inoculation						
		2-4	4	7	14	9-21	32-42	154
Analysis of combined ovary and oviduct								
Thiagarajan et al.[27]	8 log_{10} cfu/hen		28.6% (10/35) hen+					
Keller et al.[25]	8 log_{10} cfu/hen		39.4% (26/66) hens+					
Separate analysis of ovary and oviduct								
Keller et al.[20]	8 log_{10} cfu/hen	100% (6/6) ovary+; 67% (4/6) oviduct+		33% (2/6) ovary+; 13% (1/8) oviduct+		0% (0/33) ovary, oviduct+	4.2% (1/24) ovary, oviduct+	
Gast and Beard[31]	9 log_{10} cfu/hen			70% (14/20) ovary+; 60% (12/20) oviduct+	4% (1/24) ovary+; 13 (3/24) oviduct+			8% (3/40) ovary+; 5% (2/40) oviduct+
Gast[24]	4 log_{10} cfu/hen				0% (0/40) ovary, oviduct+			
	6 log_{10} cfu/hen				10% (4/39) ovary+; 5% (2/39) oviduct+			
Timoney et al.[28]	6 log_{10} cfu/hen		67% (2/3) ovary+; 100% (3/3) oviduct+	100% (3/3) ovary+; 67% (2/3) oviduct+		0% (0/5) ovary, oviduct+	0% (0/10) ovary, oviduct+	

[a]Hens were dosed with SE, sacrificed, and the reproductive organs removed for analysis of SE. Blank cell indicates no sampling.

SE colonization of the ovary

To investigate specific areas of SE colonization within the hen reproductive tract, SE was cultured from subdivided hen ovarian and oviduct tissue. Okamura et al.[32] found the ovarian preovulatory follicular membrane (capillary-rich sac surrounding the yolk within the ovary) was frequently SE positive 87.0% (21/24) suggesting contamination of the yolk could be a frequent event. However, Thiagarajan et al.,[26] who separated the follicular membrane from the yolk, found 57.0% (8/14) positive follicle membranes but only 21% (3/14) yolks (with vitelline membrane) from these follicles were positive. This suggests SE colonization of the ovary does not necessarily result in yolk contamination (see textbox). Therefore this risk assessment believes that even though the ovary can be colonized frequently, actual yolk contamination (*Ey*) will be less frequent.

SE colonization of the ovary and egg yolk contamination (*Ey*). Experiments isolating SE from the hen ovary demonstrate the ovary can be frequently contaminated.[20,21,28,32] However, Thiagarajan et al.[26] demonstrated that when yolks were removed by cutting open the follicle and letting the yolk fall into a container, the follicle membrane was more frequently SE-positive than the yolk, suggesting even though components of the ovary are SE-infected in a high percentage of hens, the yolk and the vitelline membrane appear to be infected at a lower frequency.

Thiagarajan et al.[26] suggests an explanation for this apparent contradiction. SE can contaminate the granulosa cells of the follicle membrane. During ovulation, the follicle stigma ruptures, releasing the yolk, surrounded by the vitelline membrane, into the oviduct. At this point, SE colonized/invaded granulosa cells could "slough off," onto the yolk,[26] perhaps resulting in contamination of the vitelline membrane (*Ev*) or internal yolk contents (*Ey*). This would explain the high frequency of observed ovary infections, but low frequency of fresh inner yolk contents contamination by hen infected with SE.[33]

SE colonization of the oviduct

When the oviduct was subdivided based on functionality into the infundibulum, magnum, isthmus and uterus,[c] investigators demonstrated SE positive cultures at relatively similar frequencies throughout the oviduct (Table B6).[20,32,34] However, Keller et al.[20] found the frequency of SE positive cultures from the upper magnum (secretes albumen) was greater than any other oviduct tissue (15% vs. 2.5-5%) in 1 of 3 experiments using a different hen breed.

General colonization of the oviduct implies a greater likelihood of albumen contamination far from the yolk (*Eaf*) contamination over albumen contamination close to the yolk (*Eac*) as the majority of albumen is composed of outer albumen and exposed to the oviduct for longer time periods. However, as Keller et al.[20] found, specific areas of the oviduct could be preferentially colonized depending on such factors as hen breed and SE strain. Preferential colonization of the upper magnum would probably lead to more *Eac* colonization (see textbox). This is important as the location within the egg where SE is deposited could determine the frequency and magnitude of growth.

[c] The oviduct is divided into four section based on function. The infundibulum is the oviduct opening. The magnum and the isthmus provide albumen and the inner shell membranes for the egg, respectively. The uterus lays down the outer shell.

TABLE B6. LEVELS OF SE COLONIZATION WITHIN THE HEN REPRODUCTIVE TRACT.[32,34]

Tissue	3[a]	6	8.25[d]	9	12
Ovary	0.8[b] (2/5)[c]	0.4 (1/5)	E[e]-4.3 (9/9)	1.4 (2/5)	4.2 (5/5)
Infundibulum	1.0 (2/5)	0.6 (1/5)	0-3.7 (2/9)	1.2 (2/5)	4.0 (5/5)
Magnum	1.5 (2/5)	0.6 (1/5)	0-5.2 (2/9)	0.6 (2/5)	3.7 (5/5)
Isthmus	0.6 (1/5)	0.4 (1/5)	0-4.5 (5/9)	2.1 (2/5)	4.5 (5/5)
Uterus	0.8 (2/5)	0.6 (1/5)	0-4.7 (1/9)	0.4 (1/5)	4.0 (5/5)

[a]Age of hen (months) when SE inoculated.
[b]Log_{10} SE/g.
[c]Positive samples of total assayed.
[d]Okamura et al.[32,35]
[e]SE detected below enumerable level.

Levels of SE colonization of the ovary and oviduct

Okamura et al.[32] and Hassan and Curtiss[34] measured SE levels within 4 functionally divided oviduct sections. These data indicate SE contamination of the oviduct can be considerable and extend the length of this organ. These data also suggest the hen's age affects the level of SE within oviduct tissue as older hens are more heavily colonized by SE (Table B6).

SE Within the Oviduct Likely Predicts Where SE is Initially Deposited Within the Egg

Contamination of the infundibulum, the opening to the oviduct, could yield *Ev* infection. This site is where fertilization of the ovum (yolk) takes place, suggesting intimate contact with the yolk vitelline membrane. The yolk resides in this location for a half hour after which it moves to the magnum, where it travels from upper to lower magnum (3 hrs). Within this organ, dense albumen is first deposited about the yolk, then thin albumen, followed by dense albumen and thin albumen. Infection of the upper magnum could lead to *Eac* contamination and even *Ev* contamination just as the yolk enters this organ. However, as the majority of the albumen's volume would constitute an area that could harbor *Eaf* infections, infection within the magnum would likely lead to more *Eaf* infection compared to *Eac* or *Ev*. The yolk then moves to the isthmus, where the two soft-shell inner membranes are laid over the albumen (1 hr). At this point, SE could contaminate the inner shell membranes leading to *Es* infection (see next section). *Eaf* infection could occur at any point prior to complete deposition of inner shell membranes. The yolk then moves to the uterus where the outer shell and cuticle are deposited (20 hrs). The uterus then moves the egg into the vagina followed by the cloaca. This latter organ is where the reproductive system joins the digestive system. The vagina and cloaca can be colonized by SE due to the proximity to the colon, potentially leading to SE shell contamination (*Ep*). *Ep* infection, as discussed below, could occur after complete shell deposition until the egg is laid. The egg then passes through the vent, the opening that serves for both excretion and egg laying. Therefore, depending where SE is located within the oviduct, this might dictate the incidence of *Ey*, *Ev*, *Eac*, *Eaf*, *Es* and *Ep* contamination.

Summary of SE Colonization of the Ovary and Oviduct

The data suggest both the ovary and oviduct can be heavily contaminated with SE (Table B6). Simply having ovary positive status does not predict egg contamination (see textbox).[20,24,25] The prevalence of hen colonization by SE diminishes over time to below detectable levels in the ovary and oviduct. It appears SE reproductive tract colonization can be below the level of detection, yet could still contain sufficient numbers of SE to contaminate an egg internally. The data also suggest different sites of infection within the oviduct lead to various SE localization within the egg.

ESTIMATING THE PERCENTAGE OF SE-POSITIVE EGGS BY TRANSOVARIAN INFECTION

To estimate the percentage of SE-positive eggs produced by SE-infected hens, this assessment identified studies that had investigated the number of SE-positive eggs being produced by hens known to be SE-infected. Studies were identified that followed infected hens for four weeks and one study for eight weeks. As the kinetics of infection, i.e. persistence of the organism within the hen, and how this relates to continued SE-positive egg production is unclear, this 8 week study was useful to reveal the pattern of SE-positive egg production over 8 weeks. The percentage of SE-positive eggs produced in this study is assumed to be the percentage of SE-positive eggs produced by a SE-positive non-molted hen at any one moment. Data analysis and support for this percentage is discussed below.

Typically the course of an SE infection in a group of experimentally inoculated hens begins with a large frequency of birds fecally positive for SE. Depending on the dose of the inoculation, these birds can quickly mount an antibody response that peaks within 1-2 weeks. The majority of SE-positive eggs are produced during this time. Once the antibody response has been established, fecal shedding of SE and production of SE-positive egg frequency decreases. These observations strongly suggest formation of an immune response is important for reduction of internally colonized SE and production of SE-positive eggs. Gast and Beard[21] and Gast,[24] utilizing 9 $\log_{10}$ and 6 $\log_{10}$ cfu of SE respectively to experimentally inoculate hens, demonstrated the majority of SE-positive eggs was produced within the first 2 weeks post-inoculation for all hen age groups age 62, 37 and 27 weeks). Very few eggs were produced within the following two weeks.

Bichler et al.[5]

The study conducted by Bichler and colleagues was unique in that it examined SE-positive eggs produced by SE-positive hens over an extended time period, eight weeks. SE infection was identified within four egg compartments, outer shell, inner shell membranes, albumen and yolk. The SE inoculum dose administered to hens would be expected to be sufficient to infect all hens. Eggs were examined upon lay and recovery methods to isolate SE from egg compartments were acceptable. Also, the hen serum antibody response and the SE fecal carriage were monitored during the course of the infection. Naturally infected hen studies were not used to identify the percentage of SE-positive eggs because of such unknown factors as the prevalence of SE infection within the flock and the presence of other *Salmonella* spp.

To estimate the percentage of SE positive eggs by transovarian infection, this risk assessment used the experimentally inoculated hen study by Bichler et al.[5] (see textbox). Twenty-five week old white leghorn hens were inoculated with 10 $\log_{10}$ cfu. Following inoculation, each egg produced by treated hens was cultured for SE within the albumen, yolk and the inner shell membrane compartments. This latter compartment, the inner shell membranes (IS), is located just beneath the outer shell and can be infected by transovarian infection. This compartment therefore represents an internal infection site within an egg and was used in tallying the total SE-positive eggs. The IS contamination event (*Es*) is discussed below. Based on contamination of the albumen, yolk and IS, 52% (32/61) of the eggs were internally contaminated with SE during week 1. This percentage fell to 4% (22/531) SE-positive eggs during the remaining 7 weeks (Table B7). The average of SE-positive eggs over the 8 weeks was 54 SE-positive eggs divided by 592 total eggs or 8.615% (Table B7). This number was derived from published data[5] (Table B1). Therefore, this risk assessment will use 8.615% as the percentage of SE positive eggs by transovarian infection.

TABLE B7. ESTIMATING THE PERCENTAGE OF SE-POSITIVE EGGS.[5]

	Week 1[a]	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total
Albumen+	28	1	0	0	0	0	0	0	29
Yolk+	28	2	1	2	0	2	3	2	40
Albumen and Yolk+	25	0	0	0	0	0	0	0	25
Inner shell membrane only[b]	1	0	2	0	5	0	1	1	10
Total (%) SE-positive eggs	32 (52)	3 (4.9)	3 (3.7)	2 (2.4)	5 (6.0)	2 (2.3)	4 (4.9)	3 (6.0)	54 (8.6)

[a]Weeks post-inoculation.
[b]See SE inner shell membrane contamination section.

Kinetics of SE-positive Egg Production by Transovarian Infection

To predict SE-positive egg production post-8 weeks, the trend of the infection and SE-positive egg production was examined over the 8 weeks. Fifty-two percent of the SE-positive eggs were produced within the first 7 days. This dropped to a steady rate of about 4.1%. The drop in the percent of SE contaminated eggs after 7 days was preceded by a peak antibody response that declined 17 days post-inoculation. This suggests the immune response influenced the frequency of SE-positive egg production. By 8 weeks, only 43% of hens still had detectable antibody responses. This serum antibody decline was followed by an increase in positive cloacal[d] samples, suggesting with the decline of the antibody response, SE could more vigorously colonize the hen's intestines. This could serve to infect naïve hens as well as re-infect other hens by dissemination into the environment. This increase in cloacal positive samples was not followed by an increase in SE-positive eggs by 8 weeks.

The data presented above suggest a pattern of increased SE-positive egg production immediately after SE exposure, followed by a period of lower SE-positive egg production. It is unknown if this trend would extend beyond 8 weeks, as the frequency of SE-positive eggs remained steady without further decrease from 2-8 weeks (Table B7). This risk assessment is unable to predict the percent of SE-positive eggs produced following 8 weeks. However, the data do suggest that a cycling of SE infection might occur within a flock (see textbox). That is, even though a decrease in the immune response did not result in an increased frequency of SE-positive eggs by 8 weeks, it did suggest that 57% of the hens at the end of this experiment would be able to disseminate SE into their environment due to their lowered serum antibody levels. Newly infected hens, as was seen by Bichler et al.[5] produced SE-positive eggs at a high rate (52%). Therefore, this risk assessment will use 8.615% as the percentage of SE-positive eggs produced by an infected hen by transovarian infection at any one moment.

[d] The hen cloaca is located beneath the vagina and above the vent (an opening that serves for egg laying and excretion). This organ is where the reproductive system joins the digestive system.

Infection Cycling in Naturally Infected Flocks

The concept of cycling of SE infection within a flock is supported in part by studies of naturally SE-infected flocks. Humphrey et al.[36] observed hens typically laid SE-positive (SE+) eggs in a temporal pattern, suggesting a clustering effect of SE+ egg production. Three SE+ eggs were laid between Feb. 15-17, and 5 SE+ eggs were laid between Mar 26-28. All hens produced only one SE+ egg, except for one hen that produced 2 SE+ eggs corresponding to those dates. In addition, single SE+ eggs were detected sporadically from three hens between the start and end of the experiment (Mar. 12, Apr. 7, 16). The time between the two observed clusters is 41 days (6 weeks). Clustering could represent recent infection in hens or re-infections that resulted in SE+ egg production due to the lack of a quick adaptive immune response. They could also represent times when hens are more stressed and therefore more susceptible to SE primary infection, or re-infection or low level colonized hens unable to contain equilibrium with SE due to stress. Stress due to production could have a synchronizing effect on SE+ egg production.

These data may reflect a natural cycling of transmission/infection, where more SE+ eggs will be produced by a flock at high frequency, followed by a period of sporadic SE+ egg production. Therefore, these naturally infected flock data support the possibility that the frequency of hens producing SE+ eggs will be increased during specific times. However, they do not support, nor do they negate that SE+ egg producing hens will produce SE+ eggs at a greater frequency.

Data Analysis for Estimating the Percentage of SE-positive Eggs by Transovarian Infection

Data from Bichler et al.[5] were used to estimate that 8.615% of eggs at lay will be SE-positive from transovarian infection. These data were collected up to 8 weeks post-inoculation of hens and include infection in the albumen, yolk and the inner shell membranes. In the first week, a relatively high percentage (52%) of infected eggs was observed. The uniformity assumption implicitly made is that at any time, 1/8 of the infected hens (over an 8 week period) will be just recently infected and laying (potentially) a high percentage of infected eggs. At the same time, this assumption suggests the other $7/8^{th}$ of the hens will not be laying a larger percentage of eggs (4.1%). Furthermore, the percentage of positive eggs was not decreasing for the later 7 weeks, thus it is not possible to guess or extrapolate the time when the percentage of infected eggs would be negligible. For modeling purposes, 8.615% (based on 54 positive results from 592 eggs tested) is assumed. Uncertainty of this percentage is determined assuming that these results were generated from a trinomial distribution, albumen, yolk and inner shell membrane, with $n = 592$.

Molting

After estimating the percentage of transovarian infected SE-positive eggs from SE-positive non-molted hens, the percentage of SE-positive eggs from SE-positive molted hens by transovarian infection was estimated next. Forced molting is believed to increase the frequency of SE-positive eggs produced by an SE-infected flock. As this is a fairly common practice, this population of molted flocks might produce an increased risk to the consumer. To account for this, weekly molting factors were determined and applied to the percentage of SE-positive eggs produced per week from molted flocks for 10 weeks. A discussion of the effect of molting on hens and role of the immune system in molting is given to provide an understanding of how eggs might be more frequently contaminated by molted hens. This is followed by application of these data to

modeling the effect of molting on the percentage of SE-positive eggs produced by an SE-positive flock.

Increased SE egg contamination by molted hens

As laying hens age egg production and quality decreases. Industry producers impose a forced molt on hens that results in increased egg productivity and decreased hen mortality compared with non-molted hens of the same age. Though there are many ways to experimentally induce molting, feed and water withdrawal including light manipulation and special molting diets are typically used.

Though this practice rejuvenates egg production rates and quality, experimentally and naturally infected hen studies suggests that molted hens are more susceptible to SE infection and produce more SE positive eggs post-molt (Table B8). As molted hens represent a substantial portion of the egg producing hens, this risk assessment has considered the effect of molting on the production of SE-positive eggs by transovarian infection.

TABLE B8. EVIDENCE FOR INCREASED SE-POSITIVE EGGS BY MOLTED HENS.

Publication	Study type	% SE-positive eggs by non-molted hen	% SE-positive eggs by molted hen
Holt and Porter[15]	Experimental oral inoculation	0 (0/13)	18 (2/11)
	Experimental oral inoculation	0 (0/105)	2 (3/153)
Holt and Porter[14]	Contact exposed to inoculated hens	0 (0/53)	1.6 (2/124)
Schlosser et al.[3]	naturally infected	0.02 (14/67000)	0.05 (39/74000)

SE infection susceptibility of molting hens

Molted hens are more susceptible to SE intestinal colonization than their non-molted counterparts as determined by oral inoculation studies with varying level of SE (see textbox).[14,15] These data suggest molted hens are more likely to disseminate SE into their environment. Molted hens are also more susceptible to SE infection by contact exposure to experimentally infected hens and can be infected by aerosol transmission.[15] This suggests transmission of SE among molted hens would be more rapid than among non-molted birds implying increased SE-positive egg production by molted hens could be due in part to greater within-flock prevalence.

Histopathology of molting hens

Histopathology of infected tissue from molted hens was more severe compared with tissue from non-molted hens. Histological examination of the gastrointestinal tracts of molted SE-infected hens revealed more frequent and severe epithelial cells inflammation of the colon and ceca compared with non-molted SE-infected hens.[15,37] This increased inflammation and intestinal tissue damage could allow more frequent access of SE to extra-intestinal tissues, such as the ovary and oviduct. These data suggest for the increased SE-positive egg production of molted hens.

Cellular immunity of molting hens

To study the relationship between the immune system and molting, researchers investigated varying aspects of the hen immune system. A series of 1992 papers published by the USDA Agricultural Research Services (ARS) suggest the cell mediated branch of the immune system might be impaired in molted hens. This part of the immune system is critical in activating type 2 thymus dependent B-cells to produce antibodies, stimulating macrophage mediated destruction of extracellular and intracellular pathogens, and activating cytotoxic CD8+ T-cell mediated intracellular pathogen destruction. Holt[38] reported a statistically significant decrease in the numbers of a critical set of T-cells in the serum, CD4+ T-cells, 3 days after feed removal; however, serum CD8+ T-cells were not different from controls. CD4+ T-cells are a central part of cellular immunity suggesting that this branch of the immune system of molting hens is impaired. Holt[13] and Holt and Porter[15] demonstrated the delayed type hypersensitivity (DTH) response was depressed in molted hens 3 and 7 days post-feed removal. This immunological reaction is mediated by CD4+ T_H1 T-cells. CD4+ T-cells differentiate into T_H1 and T_H2 T-cell subtypes upon antigenic stimulation. Differentiation into T_H1 cell subtype results in macrophage stimulation and recruitment to the site of infection as well as B-cell stimulation. Differentiation into T_H2 cell subtype results in a B-cell dominated antibody response. The results of the DTH experiment suggest that T_H1 cells are depressed in molted hens; however, this does not negate a role for T_H2 cells. T_H1 cells are involved in controlling bacterial intracellular infections and thereby molting hens might be more susceptible to infection due to this attenuated immune compartment. *Salmonella* spp. are capable of growing within the vesicles of macrophages. These intracellular pathogens survive because the vesicles they occupy do not fuse with the macrophage lysosome, a vesicle containing antimicrobial agents. T_H1 cells can activate the macrophage to induce vesicle and lysosome fusion, thereby increasing the likelihood of pathogen killing. At the same time, the macrophage activates other antimicrobial mechanisms and the $T_H1$cell release cytokines that attract more immune cells to the infection site. The role of T_H1 cells in mediation of intracellular bacteria suggests the increased susceptibility and pathology associated with SE infection in molting hens might be a direct consequence of depressed T_H1 numbers or function during the molting process. However, even though T_H1 cells are involved in generating an antibody mediated response, T_H2 cells are the major helper cells responsible for antibody production. Therefore, molting might not greatly affect the serum antibody response to SE.

Summary of Molting and the Hen Immune Response

In general, molted hens produce a higher frequency of SE-positive eggs than do non-molted hens. Molted hens are more susceptible to SE infection by contact exposure and experimental inoculation than their non-molted counterparts. Molted hens in a production setting will likely be more susceptible to SE infection and re-infection. Therefore, the percentage of SE-positive positive eggs produced by SE-infected molted hens by transovarian infection is increased by a modifying factor to account for molting. Because molting appears to increase the risk of SE exposure to the consumer, this risk assessment will account for this variable. Application of the molting factors is given in the following section.

DATA ANALYSIS OF MOLTING

Percentage of Annual Molted Flocks

Egg production facilities may or may not molt their hens. To identify the percentage of annual percentage of molted hens in the U.S., this risk assessment used data reported in the 1998 FSIS SE risk assessment.[4] The percentage of flocks that are molted was assumed to be 22%.

The definition of molted hens as determined by USDA-NASS is unclear. Therefore, the period hens will be considered molted is 10 weeks. After 10 weeks, hens will no longer be considered molted for the purposes of determining risk. Using the uniformity distribution assumption, it is assumed that 10% of the molted flocks will be producing SE-positive eggs for each of the 10 weeks, i.e. 2.2% of all flocks will be molted and considered to be producing a greater frequency of SE-positive eggs each week for 10 weeks.

Effects of molting flocks on percentage of SE-contaminated eggs

To determine the increase of contaminated eggs associated with molted flocks, data from the Pennsylvania *Salmonella* Enteritidis pilot project are used.[3] This study showed that molted hens produced a greater frequency of SE-positive eggs compared to non-molted hens. The percentage of SE-positive eggs was greater for 10 weeks post-molt. This increase was negligible following 10 weeks up to 20 weeks. The effect of molting on SE-positive egg production was not investigated post-20 weeks. Therefore, for the purposes of this risk assessment, a variable molting factor was applied weekly for 10 weeks to a recently molted flock (22% of flocks). Analysis for determining this variable molting factor and how it is applied is given below.

TABLE B9 DATA USED TO DETERMINE MOLTING EFFECT ON PERCENT SE POSITIVE EGGS.[3]

Molt Type	Range of Weeks	No. Flocks	No. Eggs Tested	SE-positive
Pre-	-20 to -16	3	7,000	4
Pre-	-15 to -11	9	16,000	1
Pre-	-10 to -6	12	23,000	4
Pre-	-5 to 0	12	21,000	5
Post-	0 to 5	6	9,000	13
Post-	6 to 10	8	19,000	13
Post-	11 to 15	9	18,000	2
Post	16 to 20	10	28,000	11

Let $p(t)$ be the percentage of contaminated eggs, as a function of time. There does not appear to be a clear pattern of the percent positive eggs as a function of weeks before molting. Consequently for the purposes of modeling, it is assumed that $p(t) = p(0)$ for $t < 0$. Various functions can be used to describe $p(t)$; a desirable function would be one that asymptotically approaches $p(0)$ as t 6 4 and, for small t, is not "too" large. A function that fits this description is:

$$f(t) = \frac{e^{b+ct}}{1+e^{b+ct}} + a \qquad \text{(B5)}$$

for $t > 0$, where a, b, and $c < 0$ are parameters, whose values are to be estimated from the data in Table B9. The parameter 'a' is an estimate of $p(0)$ so that $f(0)$ is set equal to a. Nonlinear regression was performed using the number of positive eggs as the dependent variable, assumed to be distributed as a binomial distribution with parameter n and $f(t)$, where n is the number of eggs tested. The independent variable is the average of the two times defining the range, given in Table B9. Regressions also were performed using related functions, such as using $\ln(t)$ instead of t in Equation B5, or assuming $f(t) = ag(t)$ where $g(t)$ is a function, but the loglikelihood was slightly greater for the function described by Equation B5 and the ratio of $p(t)/p(0)$ was generally the smallest from among those derived from other functions considered. The estimated values of the parameters, standard errors and correlation matrix are given in Table B10.

TABLE B10 ESTIMATES OF VALUES OF PARAMETERS DEFINED IN EQUATION B1, STANDARD ERRORS AND CORRELATION MATRIX, ESTIMATED WITH 5 DEGREES OF FREEDOM.

	a	***b***	***c***
Estimate	0.000226	-6.0987	-0.2302
Standard Error	0.000054	0.4843	0.0953
a	1.0000	0.2255	-0.4824
b	0.2255	1.0000	-0.8192
c	-0.4824	-0.8192	1.0000

Figure B2 is a graph of the logarithms of the observed percentages and the percentages predicted using Equation B1 and the results from the nonlinear regression versus logarithm of the number of weeks post-molt (where ln(0) is assigned a value of -2). Figure B3 presents the predicted ratios, $p(t)/p(0)$, of the percentages of SE-infected eggs for molted versus non-molted flocks versus the number of weeks post-molt.

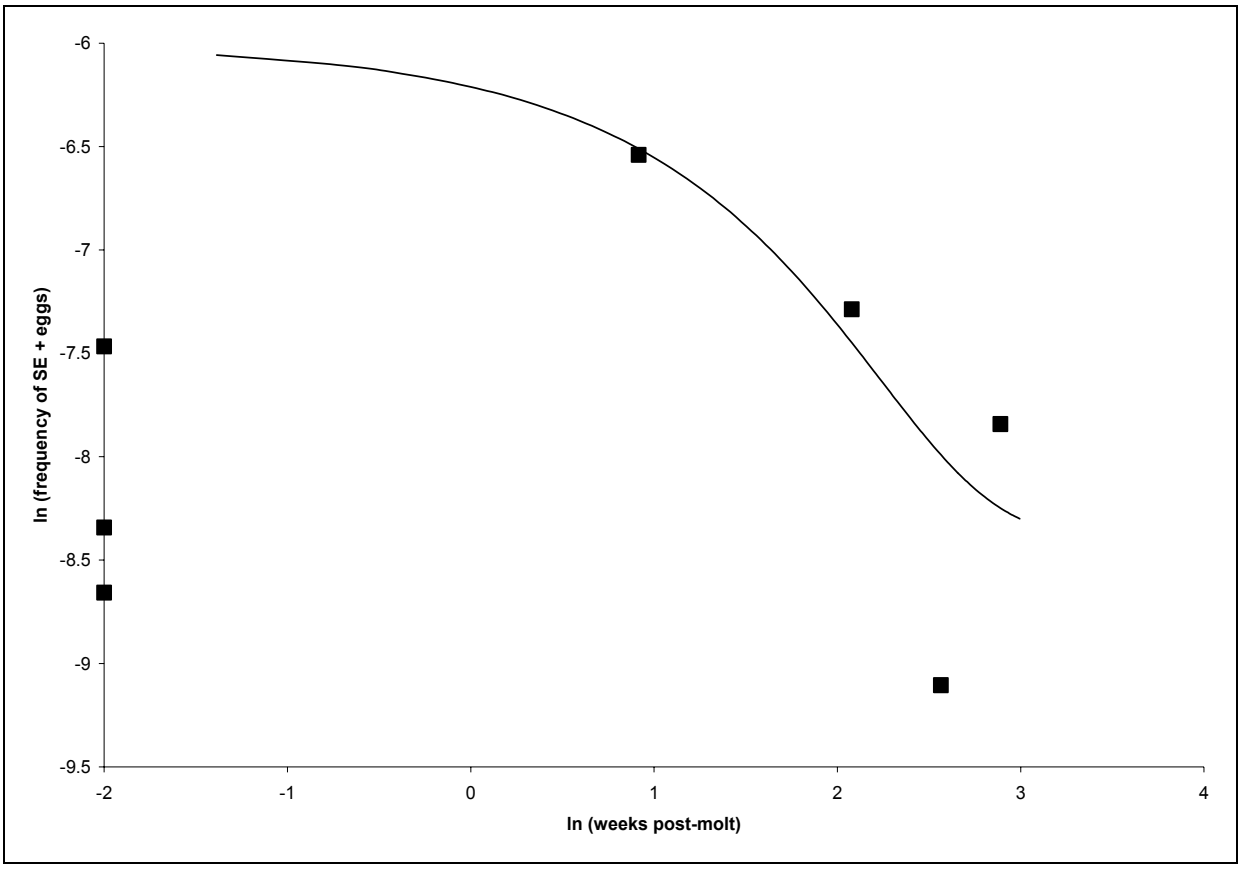

FIGURE B2 LOGARITHMS OF THE OBSERVED AND THE PREDICTED PERCENTAGES PREDICTED VERSUS THE NATURAL LOGARITHM OF THE NUMBER OF WEEKS POST-MOLT (WHERE LN(0) IS ASSIGNED A VALUE OF -2).

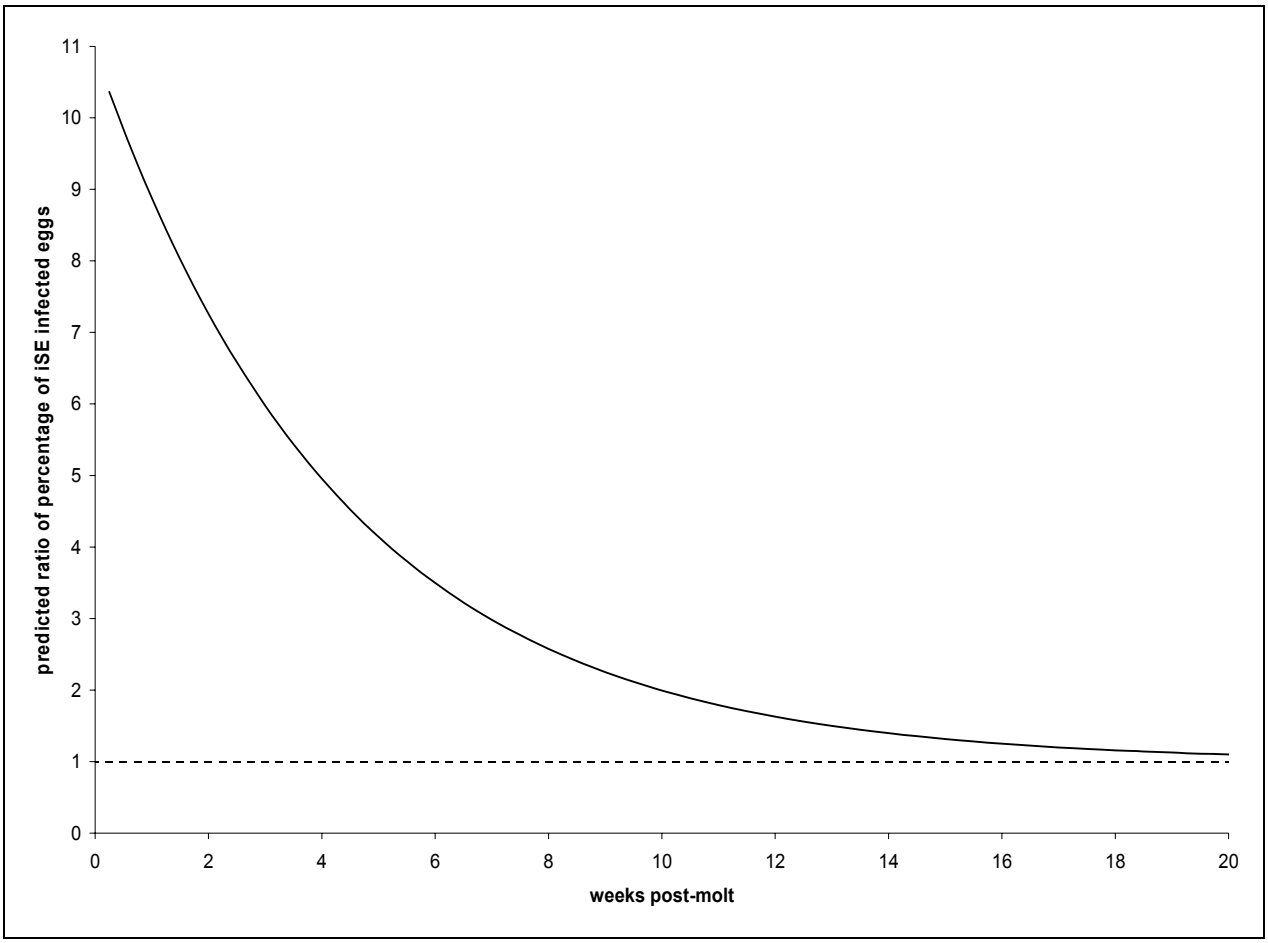

FIGURE B3 PREDICTED RATIOS OF THE PERCENTAGES OF SE-INFECTED EGGS FOR THE RATIO OF MOLTED AND NON-MOLTED FLOCKS VERSUS THE NUMBER OF WEEKS POST-MOLT.

Molting Factors

From the above figure, for just recently molted flocks, the percentage of positive eggs increases by a factor of about 10 for the first week over what would be expected for flocks that are not molted. This factor decreases weekly and is not considered past 10 weeks for purposes of determining risk.

Each weekly molting factor as determined by Figure B3 will not be applied uniformly to the 8.615% average of SE-positive egg produced by SE-infected hens over 8 weeks.[5] As mentioned in 'Estimating the percentage of SE-positive eggs by transovarian infection' section above, 52% of SE-positive eggs were produced during the first week of infection, followed by an average of 4.1% for the next 7 weeks of infection. The data are reprinted here in Table B11.

The above weekly molting factors as determined by Figure B3 will be applied to the weekly percentages in Table B11. For example, 4.9% SE-positive eggs were laid during week 2 of infection, corresponding to a molting factor of ca. 7.5 (Figure B3). Therefore, 37% of the eggs produced by molted hens will be SE-positive during the second week post-molt and the second week of infection. In addition, hens that are in their forth week of infection and producing 2.4%

SE-positive eggs, for example, and 1 week post-molt (molting factor of 10), will be considered to produce 24% SE+ eggs (2.4 x 10).

TABLE B11. ESTIMATING THE PERCENTAGE OF SE-POSITIVE EGGS.[5]

	Week [a] 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Total
Total (%) SE-positive eggs	32 (52)	3 (4.9)	3 (3.7)	2 (2.4)	5 (6.0)	2 (2.3)	4 (4.9)	3 (6.0)	54 (8.6)

[a]Weeks post-inoculation.

Because there cannot be more than 100% SE-positive eggs of the egg produced by any one molted hen, then the factor of 10 cannot be directly applied to the percentage SE-positive eggs produced by SE-positive hens during the first week (52%) of infection. Therefore, 100% of the eggs produced by molted hens will be SE-positive during the first week of infection and molt.

Molted hens and egg shell penetration

A molting factor will not be applied to the percentage of SE-positive eggs produced by egg shell penetration (*Ep*). We know of no data with which to determine the effect of molting on the prevalence of SE and other *Salmonella* spp. on eggshells.

Fraction of internal egg contamination sites

Transovarian infection results in deposition of SE within the egg. Depending on where SE was located within the hen reproductive tract, SE could contaminate a range of compartments within the egg. This includes contamination of the yolk (*Ey*), the vitelline membrane (*Ev*), the albumen near the yolk (*Eac*), the albumen far from the yolk (*Eaf*), and the inner shell membranes (E*s*). The growth of SE will differ depending on where the SE is located within the egg. This has a significant impact on the likelihood of outgrowth of SE. For instance, SE deposited in the yolk (*Ey*) or on the vitelline membrane (*Ev*) will have the greatest likelihood and rate of growth compared with SE deposited within the albumen (*Eac* or *Eaf*). This section describes how the percentages of SE located in the different egg compartment compared to one another.

This section presents the model used to compute the percentages of contamination sites within SE-positive eggs, e.g. the frequency of albumen contamination (*Ea*) vs. *Ev* or *Ey* contamination. Table B12 presents a summary of these data from various experimentally inoculated hen studies that were considered. The information given includes the SE strain used in the study, hen breed, the route of hen inoculation, the properties and the numbers of eggs analyzed, the numbers of SE-positive eggs and the numbers of contaminations detected in the albumen and the yolk. Unless stated otherwise, the numbers for the latter group are assumed to

represent either vitelline membrane (*Ev*) or internal yolk contents (*Ey*) contaminations or both.[e] *Ey* contaminations are indicated only when the authors explicitly states that the interior of the yolk was being sampled.

TABLE B12 PROFILE OF INTERNAL EGG CONTAMINATION SITES.

Strain	Hen Breed	# Eggs Analyzed		Egg Age	Fraction Positive (%)	# *Ea*	# *Ey* or # *Ev*
SE6 PT13a[21]	SPF White leghorn	Oral[a]	623	Collected daily[h], held 4 d, 25°C	NR[b]	151	141
SE6 PT13a[39]			138	Collected daily (4-14 d PI), held 7 d, 25°C	22/138 (16)	NR	NR
SE6 PT13a[40]			874	collected (6-17 d PI) and analyzed daily	25/874 (2.9)	4	21
PT13a[33]			675	collected (4-22 d PI) and analyzed daily[f]	NR	NR	29 3 *Ey* only
Y-8P2[41]	Commercial White leghorn	IC[d]	231	Egg collection unstated, but assumed daily. Eggs stored 2-5 d, 4°C[g]	5/231 (2.2)	4	1 *Ey* only
		IV[c]	274		10/274 (3.5)	8	2 *Ey* only
		Oral	221		6/221 (2.7)	6	0 *Ey* only
27A[41]		Oral	314		17/314 (5.4)	6	11 *Ey* only
Bichler et al.[5]		Oral	592	collected (1-56 d PI) and analyzed daily	44/592 (7.43)	29	40
Okamura et al.[32]	White leghorn Julia	IV	43	collected (1-7 d PI) and analyzed daily	4/43 (9.3)	1	3
Humphrey et al.[29]	Naturally infected 12 free-range hens	NA	451	Collected daily. Stored 20°C, time unknown	5/451 (1.1)[e]	1	3
	Naturally infected 23 free-range hens		68		2/68 (2.94)	1	1

[a]Route of SE inoculation
[b]NR, not reported
[c]IV, intravenously
[d]IC, intracloacally
[e]Egg contents homogenized in one sample, unable to determine original location of SE within egg.
[f]Egg collected daily on weekdays and stored 1-2 d at 7.2°C for weekend.
[g]The 314 eggs were collected 1-11 d post-inoculation (PI), constituting all found positive eggs; the study was continued up to 42 d PI, yet no positive findings in the remaining 550 eggs.
[h]Eggs collected on days 1-12, 14, 16, 18, 23 30 and 37 post-inoculation (PI).

[e] Studies reporting SE yolk infection typically did not distinguish between contamination of the vitelline membrane (*Ev*) or contamination of the internal yolk content (*Ey*).

Fraction of Ey *or* Ev *eggs*

Table B12 presents evidence to support preliminary thoughts about the possible differences in contamination rates among contamination site profiles and possible causes of these differences. Several of these studies did not distinguish between infection of the vitelline membrane and internal yolk contents. Methodologically, the yolk and the vitelline membrane were cultured for SE together. Consequently, these studies were not useful in identifying the percentage of internal yolk contamination (*Ey*) eggs. To determine this percentage, this risk assessment used the studies conducted by Gast and Holt[33] and Shivaprasad, et al.[41] These studies explicitly reported infection of internal yolk contents. The eleven *Ey* contaminations reported by Shivaprasad et al. is substantially larger than the three recorded by Gast and Holt, even after taking into consideration the number of samples and different time frames post-inoculation the samples were analyzed.

The differences between these two studies may be due to: sample handling, hen inoculation dose, hen age, hen type, analytical methodologies, and/or SE strain. These issues are important to resolve as the percentages of *Ey* infection from these two studies were quite different. Both study protocols were designed to sample yolk contents for estimation of *Ey* contaminations, while minimizing potential mixing of yolk samples with albumen or vitelline membrane. As the relative risk of yolk contamination for each study would be quite different, using both studies would generate a large amount of uncertainty. There, this risk assessment has attempted to resolve these two studies by an analysis of the factors listed above. Each issue is discussed in turn below.

Sample handling

Older eggs are more prone to outgrowth of SE due to the potential for yolk membrane breakdown. Time also allows contaminating SE to migrate into other compartments. Therefore, eggs that are not quickly collected after lay and examined for where SE was deposited might not represent the initial site of contamination. This issue was examined to see if it could explain the difference between the two studies.

Consider the data from Shivaprasad et al.[41] above. The collection schedule is unclear so a daily collection was assumed. The reasoning for this is as follows: If the eggs were not collected daily and allowed to remain at room temperature for an undisclosed amount of time prior to being placed at 4^{o}C, then the higher *Ey* events observed in this study could be attributed to SE migration from the albumen to the yolk. Shivaprasad et al.[41] report the number of days post-inoculation the sample was cultured. This suggests that for this number to be meaningful, the age of the egg was known. It can be inferred from this that the hen egg depositories were checked daily and the eggs collected daily. It can also be reasonably assumed that storage at 4^{o}C would minimize migration of SE from the albumen to yolk contents. These assumptions imply the sampling protocol of Shivaprasad et al.[41] is similar to that of Gast and Holt[33] with respect to their effect on the likelihood of finding yolk positive samples. Building on this interpretation, the differences between data from Gast and Holt[33] and Shivaprasad et al.[41] need to be explained by considering other factors.