ANNEX E

Modeling Growth of *Salmonella* Enteritidis in Eggs

CONTENTS

INTRODUCTION

The human health risk from contamination of eggs with *Salmonella* Enteritidis (SE) depends upon the number of SE in an egg, which in turn depends upon the initial level of SE contamination and the extent to which SE can grow as the egg proceeds from farm to consumer. This annex describes the development of a model to estimate growth of SE in eggs. This model was derived from a series of functional relationships that rely on numerous parameters, including temperature, time, pH, *Salmonella* strain, host immune response, site, and level of initial contamination, and yolk membrane integrity. Thus, when developing a model for SE growth in eggs, all of these factors must be considered.

The model presented here is different from that of the 1998 SE risk assessment.[1] The manner in which SE grows within the egg is better understood now than it was in 1998. Data are available with which to derive SE growth rates in egg yolk and albumen. Two distinct compartments of albumen exist; these may be described as close to, and farther from, the yolk. There is evidence suggesting SE growth within albumen decreases with increasing distance from the yolk.[2] Further, the number of SE cells that the albumen can support may be substantially less than that of the yolk.[2;3]

Modeling SE growth rates in multi-compartment shell eggs is further complicated because physiological states within the egg compartments are dynamically changing and could significantly affect growth. This is illustrated by the high variability and the multi-modal character of the distribution of SE levels in experimentally inoculated eggs stored in controlled environments.[2;3] Findings from a study conducted by Humphrey and Whitehead,[2] in which eggs were inoculated with SE in the albumen, generally revealed high SE levels in eggs for which the yolks were SE-positive and low SE levels in those for which the yolks were not SE-positive. The difference was explained by supposing that cells migrated to the yolk and grew, or that the nutrients of the yolk were made available to SE in albumen, thus initiating rapid growth and migration into the yolk. We picture the event of making nutrients available to SE in eggs, resulting in a relatively large number of SE, as being caused by a weakening of the membrane surrounding the yolk thus allowing nutrients to escape into the albumen, or permitting SE to gain direct access to the nutrients of the yolk. Once the weakening begins, the duration of the growth period is believed to be relatively short. Consequently, the series of events that lead to the rapid growth is referred to as a singular event in time, here termed yolk membrane breakdown (YMB). Once YMB has occurred, yolk nutrients may be widely available throughout the egg. Up until the time of YMB, we assume that growth to large numbers of SE within the albumen could not be supported. Thus, we assume the growth rate in albumen-contaminated eggs is minimal until YMB, whereupon growth rates may increase until the number of cells approaches approximately 10 $\log_{10}$.

MODIFIED VISION FOR DEVELOPING AN SE IN EGGS GROWTH MODEL

The differences between the current model and the 1998 growth model[1] can be summarized in four main areas: (i) SE growth within the albumen; (ii) rates of relative SE growth within an egg, depending upon location or site of contamination; (iii) accounting for possible lag and stationary

phases of SE growth; and (iv) the specific modeling of SE growth within the egg, using stochastic equations. Each of these differences is discussed in turn below.

Modeling SE Growth within Albumen

Humphrey[4] previously presented a view of SE growth within eggs, which incorporated an expectation of SE growth within albumen during the first 24 hours after an egg was laid. Little SE growth was expected within the albumen in eggs older than 24 hours. This view of SE was the basis of growth modeling envisioned in the 1998 SE risk assessment.[1]

Growth within egg albumen in the first 24 hours post-lay was not modeled here. The notion that an approximate 10-fold increase in the number of SE occurs within the first 24 hours of lay, a claim we judge may be supported by hypothesizing that SE are able to utilize internal reserves of iron and to grow while the pH is neutral, may be attributed to Humphrey.[4] However, data supporting this notion were ambiguous. Specifically, the claim seems to have been based on two premises and an experimental result. The premises are that the pH in albumen increases from about 7.5 to 9 or higher within the first 24 hours after lay, and that SE cannot grow in an environment where the pH is above approximately 9. This would lead to the conclusion that SE cannot grow in the albumen in eggs more than 24 hours old. Experimental results demonstrate an approximate 10-fold increase for experimentally inoculated eggs less than 1 day old, and subsequently stored for 5 days.[2] If the two premises were true then it would follow that the 10-fold increase in SE growth had to take place within the first 24 hours after lay. However, the validity of the second premise is in question as it appears that SE can grow up to pH 9.5, [5] which is representative of albumen in eggs older than 24 hours. Thus, this risk assessment did not assume a particular phase of growth associated with the first 24 hours after lay.

Evidence exist supporting SE growth in albumen under certain physiological and experimental conditions (Table E-A1). Therefore, more potential SE growth within the albumen than had been assumed possible in the previous work cannot be dismissed. Instead of using a 10-fold increase in the first 24 hours, growth in albumen is explicitly modeled for this risk assessment.

Rates of Relative SE Growth within an Egg Versus Location of Contamination

SE are believed to grow at differing rates depending on where in the egg initial contamination occurs. Limited data are available to describe these different SE growth rates. A series of contamination events, *E*, were envisioned at different sites of the egg, or egg compartments, as shell eggs develop within the hen. These contamination events are summarized as follows: *Eaf*, contamination of albumen far from the yolk; *Eac*, contamination of albumen close to the yolk; *Ev*, contamination of the vitelline membrane of the yolk; and *Ey*, contamination of the yolk. Significant changes from the previous model[1] are that the current one includes modeling SE growth in albumen depending on site and distance from the yolk (*Eac* and *Eaf*) and on the vitelline membrane of the yolk; modeling the physiological lag phase before growth is initiated; and modeling variability and uncertainty associated with growth parameters, allowing for differences of growth for these compartments.

Accounting for Possible Lag Phase and Stationary Phases of SE Growth

Experimental growth of bacterial populations is generally described by sigmoidal curves of $\log_{10}$ counts versus time. Growth can be described to occur in three phases: lag, exponential, and stationary. The initial flat portion of the curve is described as the physiological lag phase when cells are adapting to their environment before growth can begin. Once cells are exposed to favorable growth conditions for certain periods, the exponential growth phase can begin. Physiologic and genetic limitations constrain exponential growth as the maximal population density (M) or carrying capacity of the population is approached. This limit is the stationary phase. A logistic growth assumption was used to capture the expected decreasing exponential growth rates as the levels approach M. Unfortunately, data on lag phase duration of SE in eggs are not available. Possible values for parameters used to characterize the lag phase were taken from the published literature,[6] the details of which are provided subsequently in this annex.

Stochastic Growth

A deterministic growth rate may not adequately describe growth for small initial densities of bacteria. To address this, the current model incorporated the random or stochastic nature of bacterial growth. Specifically, the distributions of the times at which changes of states occur, either of an SE cell in lag phase changing to one in exponential phase, or of an SE cell in exponential phase dividing, were assumed exponential. The details of the derivations and equations used to model this stochastic growth and its application in these risk assessments are provided in Attachment 1 of this annex. Given the description of the principle differences between the 1998 SE growth model[1] and the current one, the remainder of this annex explains the current model in detail.

Table E1 describes the models that were used to predict growth of SE for the different types of contaminated eggs. The elements of Table E1 and this basic model are described at length in three sections that follow. The derivation for the stochastic model of growth is found in Attachment 1.

TABLE E1 GROWTH MODEL SUMMARY

Description (data sources)	Distribution	Values	Uncertainty
1) Growth in albumen[7] 1) probability that eggs experience growth, *p*; 2) expected exponential growth rate *.*: when there is growth	1) *p* is constant. *.*: lognormal with mean of ln(*.*) = *m*, standard deviation of ln(*.*) = Φ; actual growth dependent on assumed ratio of lag/generation time, *rat*	See Table E5 *p* = 0.788, dependent on contamination site: *Eac p* (*Eac*) = 0.788, *Eaf p* = 0, … *p*(*Eac*)	*p* (*Eac*) based on binomial with 85 samples. *m, σ* based on 85 samples: standard error *m* = σ/(85)$^{0.5}$, normal distribution; σ^2 chi square distribution with 84 degrees of freedom. *rat*: State of Knowledge
2) Time before Yolk Membrane Breakdown (YMB)[8] 1) probability of YMB, *p*(*t,T*) as a function of time *t* and temperature *T* with parameters *d, f, g, k* 2) Probability adjustment for YMB based on ∑, *p*	1) Extreme value: *p*(*t,T*) = 1-exp(-exp(-*a(T)* + *b(T)t)*), where *a(T)* = e^d, *b(T)t)* = e^{f+gT} -*k* 2) *p* = 35/120	1) See Table E4 2) See Equation E12	1) See Table E8 for variance-covariance matrix for TMB 2) *p* based on binomial with 35 successes out of 120 observations
3) Ratio of Lag time to generation time for growth in albumen and yolk, *rat*	NA	*rat* = 1, …, 10	State of Knowledge
4) Growth in yolk and yolk membrane as a function of *e*, *f*, *b, Tmax, v,* and $w^9$990[10-14]	See Attachment 2 on stochastic process	See Tables E12 and E13	See Table E14 for variance-covariance matrix for *μ*; *M* is considered constant, State of Knowledge

SE Growth in Albumen Before Yolk Membrane Breakdown

Evidence suggests SE growth occurs in egg albumen; yet the probability of such growth occurring, and if so to what extent, is unclear. Available evidence indicates SE growth in albumen is highly variable and may strongly depend on experimental design and methodology. Selected studies are discussed below.

Derivation of values of parameters for modeling growth before YMB

To determine the amount of SE growth that might take place before YMB, it is necessary to ascertain for which eggs YMB has not occurred. This cannot be known with certainty and must be inferred from knowledge of other factors. The only possible way to determine YMB directly is to examine microscopically interior egg structure. For instance, Mytle and Chen[15] observed loss of the integrity of the vitelline membrane of SE-contaminated eggs by scanning electron microscopy. However, because microscopy is not used in most growth studies, indirect evidence has to be relied on to determine YMB. To this end, however, there are no general rules for determining a criterion for YMB. Instead, evidence, and thus the criterion for indicating YMB,

depends upon an examination of relative amounts of measured SE. A certain amount of consistency should exist between the criteria derived from different studies.[2;4;8] In part it is believed that the albumen of intact eggs can only support a certain level of SE, which may vary depending on the particular egg storage conditions. Eggs in the aforementioned studies were inoculated with approximately 500 SE (phage type 4) at various times after lay. In the study by Humphrey and Whitehead,[2] eggs were held at 20±0.5°C for 5 days after inoculation and assessed for the number of SE. Table E2 shows the percentage of intact eggs stored at 20EC for which there was more than a 3 $\log_{10}$ increase of SE.

TABLE E2 PERCENT EGGS WITH LARGE RELATIVE GROWTH.[4]

Days storage on intact eggs prior to inoculation of contents with SE	% eggs where the inoculation increased by factors that exceed $\log_{10}$ 3.0 [a]
0	2.7
7	5.0
14	4.0
21	10.0
28	35.0
42	87.0

[a]Inoculum was approximately 500 (2.7 $\log_{10}$) cells per egg. Eggs were held at 20.5°C for 5 days after inoculation. Data are mean values from up to nine separate experiments. In each experiment, 20-60 eggs were examined at each sampling point.

Humphrey[4] assumes relative growth in excess of 3 $\log_{10}$, which correlates to a final level of 5.7 $\log_{10}$ SE (i.e., 2.7 $\log_{10}$ initial concentration and 3 $\log_{10}$ growth), is evidence that YMB has begun. Humphrey and Whitehead[2] claim that levels of SE in an egg can be as high as 5 $\log_{10}$, with few exceptions, without YMB. It is unclear why Humphrey[4] or Humphrey and Whitehead[2] do not consider SE levels below 5.7 $\log_{10}$ SE indicative of YMB. Data were not provided that showed a distribution of the number of SE cells; thus, whether there were many samples with levels just below the 5.7 demarcation point (or whether the number of samples with more than 5 and less than 5.7 was also small, so that the demarcation value of 5 could have been just as reasonably chosen) is not clear. The possibility of a value of 5 is consistent with data from an experiment with a similar protocol.[8] These latter data include the levels of SE measured in eggs that were stored for a fixed number of days at fixed temperature; inoculated with 500 SE in the albumen, near the yolk; or stored for 4 days at 20EC before measurement. Figure E1 summarizes data from Humphrey.[8] The majority of eggs were found to have less than 5 $\log_{10}$ SE, but eggs with very high levels of SE (7-8 $\log_{10}$) were observed occasionally.

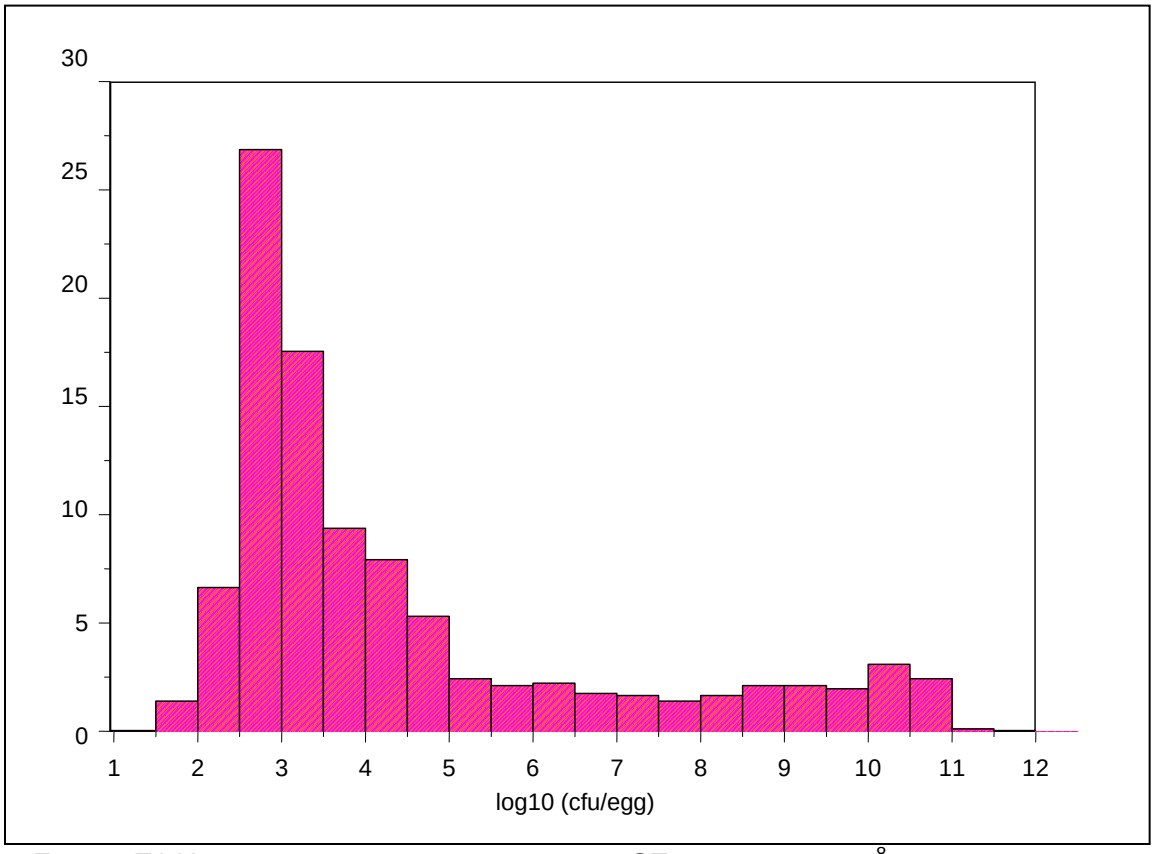
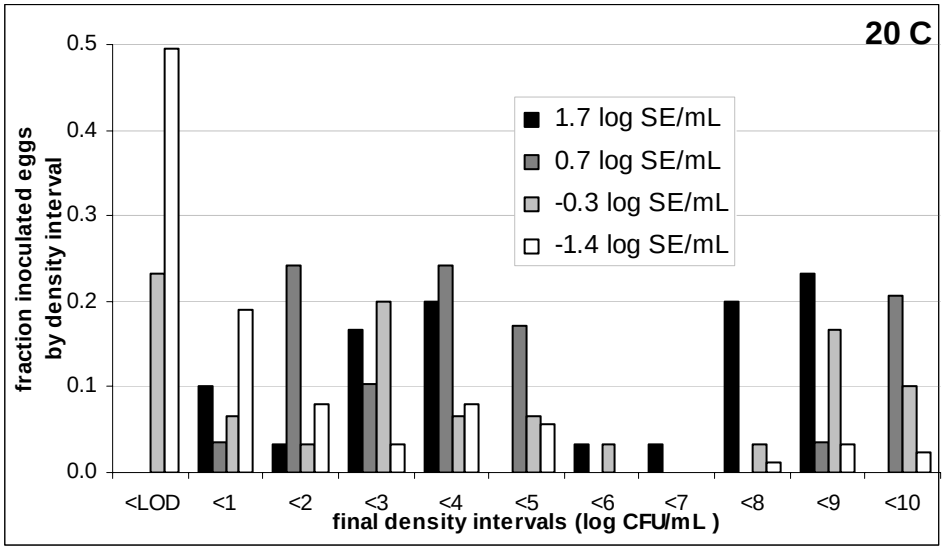


FIGURE E1 HISTOGRAM OF LOG_{10} OF OBSERVED SE CELLS PER EGG.[8]

The protocol for the study by Cogan[7] was different from that of the studies by Humphrey.[4;8] Thus, differences in the levels and the criterion for YMB were expected. Cogan[7] measured SE levels in eggs after inoculating close to the yolk (*Eac*) and storing for 8 days at 20 and 30EC. There were four inoculum target amounts: 2, 25, 250, and 2,500 SE per egg. In total, there were 330 samples. Note that the reported levels are expressed in cells per ml. The volume of an egg is assumed 50 ml. The histograms in Figure E2 summarize data from Cogan.[7]



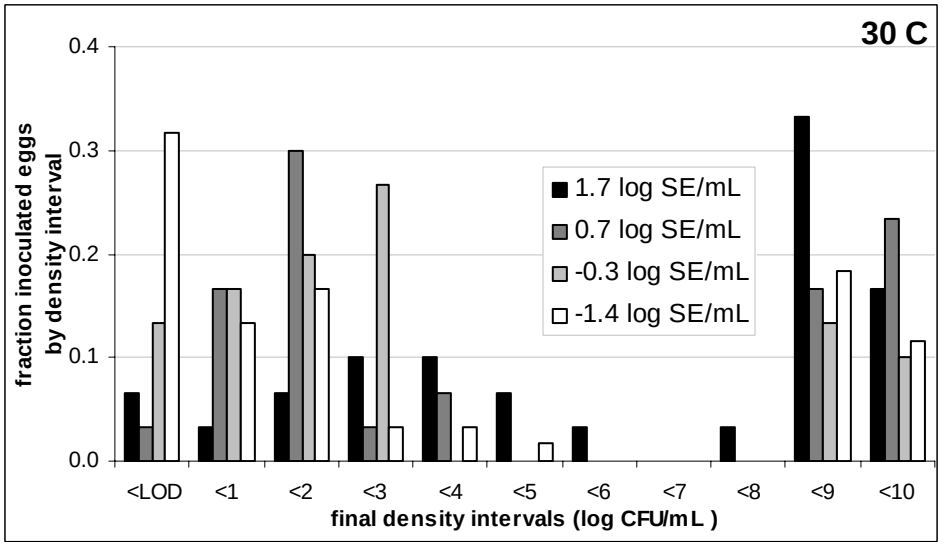


FIGURE E2 COUNTS OF SE CELLS IN WHOLE EGGS INCUBATED AT 20 AND 30°C. EGGS WERE INOCULATED WITH FOUR DENSITIES OF SE FOR 8 DAYS.[7] SE LEVELS ARE EXPRESSED PER ML ASSUMING EGG CONTENT IS 50 ML.

Data from Figure E2 are indicative of two populations of eggs: the first, consisting of the majority of the eggs studied and containing SE levels not more than about 5 $\log_{10}$ per ml, is believed to be those eggs with intact yolk membranes; the second, with SE levels between 8-10 $\log_{10}$ SE, is believed to be those eggs in which YMB has occurred. Eggs with intermediary levels of SE, i.e., between 5 and 8 $\log_{10}$/ml, were rarely observed. If rapid SE growth occurs at YMB and if the albumen by itself cannot support more than a certain level of SE, it is understandable that there would be an interval of SE levels with only a few eggs observed. Between 5 and 7.7 $\log_{10}$ SE, there were two results: 5.3 $\log_{10}$ SE/ml and 6.3 $\log_{10}$ SE/ml. For the data from Cogan,[7] it

was assumed that eggs with greater than 5.3 $\log_{10}$/mL of SE experienced YMB. This translates to about 7 $\log_{10}$ SE cells per egg.

Figure E2 also reveals the large amount of variability among SE levels within eggs assumed not to have undergone YMB. It is possible that stochastic events within the albumen took place within the egg that enabled levels of SE to increase substantially even without the occurrence of YMB. Nevertheless, this variability in the data was accounted for in the model presented here, as described below. The higher demarcation value for data from Cogan[7] data compared to data from Humphrey[4;8] could be explained by the 8-day incubation interval for eggs in the former study (which may have permitted more growth after an initial lag phase) compared to the 4 to 5 day incubations in the latter studies.

From the whole egg study by Cogan[7] the following facts may be accepted: the data represent eggs that are contaminated near the yolk (*Eac*); YMB is associated with samples that exceed a concentration greater than 5.3 $\log_{10}$ cfu/mL; substantial SE growth within the albumen can occur before YMB; the amount of growth of SE within albumen of eggs is highly variable; and there is an upper bound on the number of cells that the albumen can support, which is substantially less than the high levels seen when growth is influenced by yolk material.

The model assumes that for a certain percentage of eggs, a certain level of SE growth can occur within albumen before YMB. In turn, YMB is, for the most part, dependent on the temperature at which the egg is stored. Once YMB occurs, a lag phase allowing the cells to acclimate to the changed environment and utilize yolk nutrients follows. Then, rapid SE growth commences to the point where levels of 10 $\log_{10}$ cfu/mL per egg can be reached. The details of the model follow the presentation of a recently received dataset (below.) These data represent a potential alternative to the model assumptions adapted for these risk assessments.

Data that might support an alternative model formulation

An additional dataset on SE levels from experiments with isolated egg albumen was received from Cogan.[7] The data are expressed in SE levels per ml in isolated albumen samples that were stored for 8 days after inoculation with one of 4 possible SE levels. This is similar to the whole egg data discussed above.[7] The histogram of the data (Figure E3) presents quite a different scenario than that stated above. These data show high levels of SE, approaching 8-$\log_{10}$ cfu/ml, which corresponds to 10 $\log_{10}$ cfu/egg for isolated albumen.

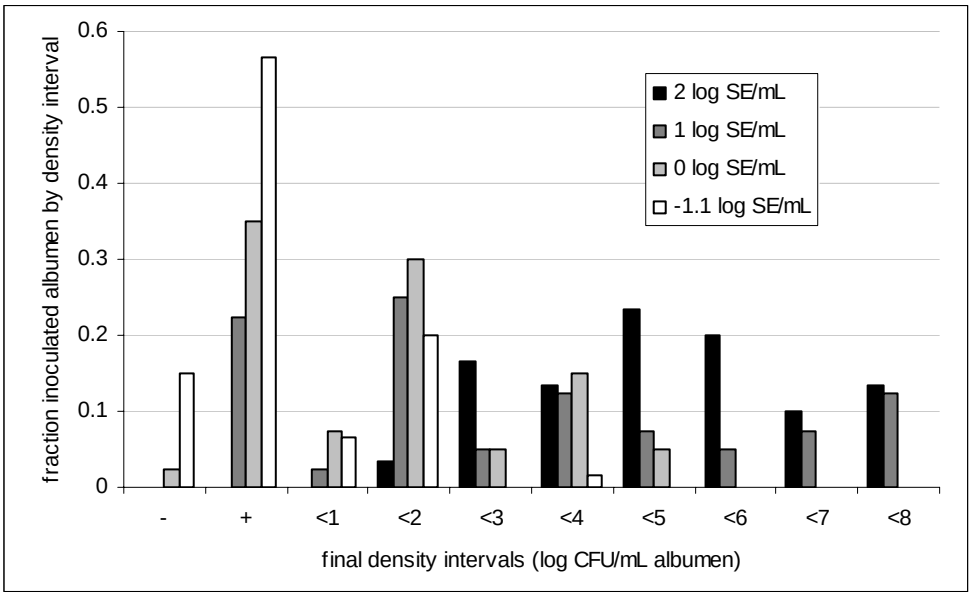


FIGURE E3 HISTOGRAM OF SE LEVELS (LOG_{10} SE/ML) IN ISOLATED ALBUMEN HELD 8 DAYS AFTER INOCULATION AT 20ºC.[7]

SE can grow in albumen, but perhaps not well (Table E-A1).[2;4;16] These results suggest that albumen can support up to 5-6 log_{10} cells/ml. Though data in Figure E3 show the likelihood of high levels of SE depends upon initial SE levels, this observation does not adequately explain how the high SE levels could have occurred. It might be that when SE reach certain levels, the likelihood of rapid growth increases more so because of the large numbers of cells, and less so because of YMB. Hence, for whole egg data,[7] few SE levels between 5 and 8 log_{10}/mL would be seen because when levels reach 5-6 log_{10} the likelihood of cells having access to the yolk nutrients is high so that rapid growth would commence and high levels of SE would be obtained shortly. If this were so, then clearly our model of YMB and the implied rapid growth that follows would be inappropriate because it does not associate these events with SE levels in the albumen. If such a model were developed from these data, it would legitimately only apply to *Eac* contaminations, since the data represents this type of contamination. However, the application of the model assumes at most a very moderate dependency on level and is assumed to apply for all contaminated eggs, including *Es* and *Ep*, with the assumption that once the yolk membrane weakens, yolk nutrients become available throughout the egg and the kinetics of rapid growth commence regardless of the number of SE present.

We are to some extent recognizing that distance of SE from the yolk may play a role in growth rate, and we are inserting, somewhat arbitrarily, state of knowledge parameters for the distribution of the times of YMB and lag phase duration, dependent upon the type of contamination. However, the above features of the model do not fully address the problem raised by data from isolated albumen.[7] We are not building a model based on these most recent data for isolated albumen.[7] This is the only research we know of that shows such high SE levels in the albumen. Because the data do not represent SE levels in whole eggs, they were not used in these risk assessments. We acknowledge that the likelihood of rapid SE growth due to access to yolk nutrients may depend on the SE levels in the egg and that, if so, there may be deficiencies in our model. More research is needed to resolve this issue.

Determination of distribution of SE growth rates within albumen before YMB

Based on the observation of large variability in SE growth, the model inputs were distributions of random variables. Specifically it was assumed that the exponential growth rate, μ, and the event of growth taking place were random variables. Another possibility would have been to assume that the lag phase duration is a random variable. Once the lag phase is completed for a cell, the cell enters an exponential phase of growth, with an exponential growth rate equal to a constant that is the same for all cells in all eggs. There is no scientific basis for choosing one possibility over the other. Research regarding the nature of the growth of SE in albumen is needed sorely to clarify this issue. Values that characterize the distributions were determined here by examining data for whole eggs,[7] which suggest the fraction of eggs with SE levels above 5.3 $\log_{10}$ to be dependent on the inoculum level (Table E2). For the higher inoculated levels, the fraction does not appear to be strongly temperature-dependent. Consequently, results from Cogan[7] were assumed to apply to temperatures between 20 and 30ºC. Using the growth model developed below, a temperature dependence on the exponential growth rate of SE may be determined.

An analysis of the data from Cogan[7] is presented below. This analysis was used to estimate values of parameters that characterize the growth curve for temperatures between 20 and 30ºC, and was assumed to apply for *Eac* eggs. Following this analysis, the assumptions for modeling growth for *Eaf* eggs and the exponential growth rates as a function of temperature are given to characterize growth of SE in an egg before YMB.

TABLE E4 FRACTION OF SAMPLES WITH MORE THAN 5.3 $\log_{10}$ CFU/ ML.[7]

	Target Inoculated Level Per Egg (cfu SE)									
Temp	**2**		**25**		**250**		**2,500**		**All**	
(°C)	*N*	*Frac.*	*N*	*Frac.*	*N*	*Frac.*	*N*	*Frac.*	*N*	*Frac.*
20	90	0.07	30	0.30	30	0.23	30	0.50	180	0.21
30	60	0.30	30	0.23	30	0.40	30	0.53	150	0.35
All	150	0.16	60	0.27	60	0.32	60	0.52	330	0.27

Eac-*contaminated eggs*

Eggs with less than 5.3 $\log_{10}$ SE cfu/mL were classified according to whether their SE populations increased.[7] When a target 2 cells were inoculated, the errors in the measurement of the numbers of cells inoculated and the measurements of the numbers of cells after 8 days were large. An accurate determination of whether SE growth occurred is difficult. Consequently, eggs inoculated with a target 2 SE cells were excluded from further analysis.

In contrast, for eggs inoculated with 2,500 cells, the possibility exists that growth kinetics would be affected by the large numbers of cells and would not be representative of the kinetics for contaminations with smaller numbers of cells. As such, eggs inoculated with 2,500 SE cells were also excluded from the analysis. The relative growth rates of SE for the remaining eggs were estimated assuming the volume of the egg to be 50 ml. The ratio of the measured SE level and the inoculated number of cells divided by 50 was thus used to estimate relative growth for SE in each egg. The distribution of the $\log_{10}$ of the relative growth, *logr*, showed a "gap" between 0.079 (20% increase) and 0.301 (100% increase). Therefore, eggs with *logr* less than 0.08 were assumed not to have had SE growth, whereas eggs with *logr* greater than 0.3 were assumed to have had SE growth.

Results from eggs considered to have experienced growth of SE cells were used to estimate the distribution of the exponential growth rates for SE in albumen of *Eac*-contaminated eggs prior to YMB. To determine the distribution, a growth curve based on a two-compartment model was assumed. Cells were initially considered to be in lag phase for some period, after which they entered exponential phase. The distributions of the duration of the lag phase and the times of cell division were assumed exponential, characterized by parameters λ, which determine the time that inoculated cells remain in the lag phase before entering the exponential phase, and by μ, the exponential growth rate. One further assumption was that when the number of cells approaches the stationary phase, i.e. close to the maximum number the system could support, the rate of growth decreases. Based on these assumptions, the differential equations that describe the number of cells, assuming a large number of cells, at a given time are:

$$\frac{dS_L(t)}{dt} = -\lambda S_L(t) \tag{E1a}$$

and

$$\frac{dS_s(t)}{dt} = \lambda S_L(t) + \mu S_e(t)(1-\frac{S(t)}{M}) \tag{E1b}$$

where $S_L(t)$ is the number of cells in lag phase; $S_e(t)$ is the number of cells in the exponential phase or beyond; $S(t) = S_L(t) + S_e(t)$; and M is the maximum number of cells the system will support

The above set of equations does not have a closed form solution. Using a slightly different formulation, however, a closed form solution has been developed.[17] Baranyi's equations are based on the differential equation

$$\frac{dS(t)}{dt} = \alpha(t)\mu S(t)\left[1 - \frac{S(t)}{M}\right] \tag{E2}$$

where $0 < \alpha(t) \leq 1$, and noting, by invoking the chain rule for differentiation, that the solution is of the form: $f(A(t))$, where $f(t)$ is the solution of the above equation when $\alpha(t) = 1$ (f is logistic) and $dA(t)/dt = \alpha(t)$. The factor $\alpha(t)$ can be thought of as determining, or controlling, the rate at which cells enter exponential phase. The solution to the above equation is

$$n(t) = n_0 + \mu A(t) - \ln\left[1 + \frac{e^{\mu A(t)} - 1}{e^{(n_{max} - n_0)}}\right] \tag{E3}$$

where $n(t) = \ln(N(t))$, the natural logarithm of the level of cells (cfu/ml) at time, t; s_0 is the logarithm of the initial $\log_{10}$ cfu/ml levels of SE suitably transformed to natural logarithm units; n_{max} is the logarithm of the maximum (5.3 $\log_{10}$ cfu/ml) levels of SE suitably transformed to natural logarithm units; and $A(t)$ is an adjustment factor for lag duration time.

One possible solution is

$$A(t) = t + \mu^{-1} \ln\left[\frac{e^{-\mu t} + q}{1+q}\right] \tag{E4}$$

where q is a parameter that characterizes the environmental and physiological states of the cells, and thus affects lag times. To determine q, a relationship reported by Ross[6] was used. Ross[6] studied many growth data sets and often found that values of the ratio of the mathematical lag time (*Rat)*, defined as the intersection of the line tangent to the growth curve at the point of maximum slope and the line $y = s_0$ and the generation time, ranged between 3 and 10. Furthermore, they were relatively constant over a wide range of exponential growth rates and lag phase duration times. Thus, it was assumed in these risk assessments that *Rat* is constant.

Using the above equations, *Rat* can be expressed as a function of q. For Equations E1a, E1b and E4, when ignoring the terms describing the growth near the stationary stage (logistic factor) and using the asymptotic line instead of the tangent line, the mathematical lags, *mathLag*, are

$$mathLag = \begin{cases} \nu^{-1}\ln(1+\frac{1}{q}), & for\ Eq.\ (4) \\ \mu^{-1}\ln(1+\frac{\mu}{\lambda}), & for\ Eq.\ (1a)\ and\ (1b) \end{cases} \tag{E5}$$

Equating the two *mathLag* values it is seen that $q = \lambda/\mu$. Thus, the ratio, *Rat*, of the mathematical lag to the generation time is equal to

$$Rat = \frac{\ln(1+\frac{1}{q})}{\ln(2)} \tag{E6}$$

Many of the data sets studied by Ross[6] were from studies using broth media. The results may not be applicable to growth of SE within eggs but they are the only data available. The cells used in these studies are assumed to have been in the stationary phase because the typical protocol involves an 18 to 24 hour or more incubation period before sample inoculation. The distribution of μ was determined by calculating μ values for each egg satisfying Equation E3, where n (8) is equal to the natural logarithm of the observed and then computing the mean and standard deviation of the values of $\ln(\mu)$. The percentage of samples that showed growth, the mean, and the standard deviation of $\ln(\mu)$ for assumed values of *Rat* are given in Table E5.

For determining the distribution of relative growth in *Eac* eggs, *Rat* was assumed a state of knowledge variable. For an assumed value of *Rat*, the distribution of μ was determined. Even though the skewness and kurtosis of $\ln(\mu)$ were slightly negative for the assumed values of *Rat*, it was assumed that the distribution of μ was lognormal. This was done to assure that positive values of μ were generated.

TABLE E5 ESTIMATED PARAMETERS OF THE LOGNORMAL DISTRIBUTION OF EXPONENTIAL GROWTH RATES ASSUMED FOR EGGS FOR WHICH THERE IS NO GROWTH OF SE. ASSUMED RATIO OF LAG TO GENERATION TIME (RAT) ARE FOR CELLS IN STATIONARY PHASE.

3	-0.234	0.477
4	-0.121	0.426
5	-0.021	0.385
6	0.070	0.352
7	0.151	0.324
8	0.227	0.300
9	0.296	0.280
10	0.361	0.262

Of the 85 samples used in the above analysis (Table E5), 78.8% of the eggs experienced growth of SE. Thus, the percentage of *Eac*-contaminated eggs that would show SE growth was estimated with a binomial distribution with $n = 85$ and $p = 0.788$ (or a normal approximation to account for the variability in the number of eggs). It was assumed that cells contaminating the eggs would have smaller lag phase durations than those used by Cogan.[7] To reflect this for a given ratio, *Rat*, it was assumed that the ratio value for the cells in the naturally contaminated eggs was ½ that of *Rat*.

Eaf-contaminated eggs

Data regarding growth of SE in *Eaf*-contaminated eggs are not available. However, growth of SE is expected to be slower in *Eaf* eggs than in *Eac* eggs.[2] The reason for this is not known, though it could be that the exponential growth rates are less in *Eaf* than *Eac* eggs; the lag phases in *Eaf* eggs are longer; or both. The following assumptions were made: for eggs which experience SE growth, the exponential growth rates of SE would be the same; the percentage, *p*, of eggs that experience SE growth would be less than or equal to that of *Eac* eggs; the lag phase duration for cells of *Eaf* eggs would be greater than that of cells in *Eac* eggs. *p* was assumed a state of knowledge variable, ranging from 0 to 78.8%. The value of *Rat* for these eggs is ¾ of the *Rat* given in Table E5.

Extension of Growth Model to Arbitrary Temperatures

At the temperatures tested by Cogan,[7] 20 and 30°C, growth of SE did not appear temperature-dependent. Therefore, it was assumed the above growth model applies for temperatures between 20 and 30°C, and that the midpoint of this range, 25ºC, was the best point estimate of a single temperature. The uncertainty attending the temperature was modeled in the risk assessments by a uniform distribution between 20º and 30ºC.

The two temperatures tested by Cogan[7] were not deemed sufficient to describe growth over the full range of potential growth temperatures. However, the exponential growth rates derived above apply for a temperature, *T*, selected from this distribution. Thus, to determine the

exponential growth rates of SE for other temperatures, data from measurements of levels of SE on the surface of the yolk (vitelline membrane) over time[16] were used. The relationships of the exponential growth rates and temperatures that were derived from these data were used to determine the relationship between exponential growth rates in albumen and temperature, as explained below.

Gast and Holt[16] performed growth experiments by inoculating the vitelline membrane with SE and storing eggs at various temperatures. An important point is that growth was observed up to a maximum level of approximately 6 log10 cfu/ml. This is, perhaps coincidentally, near the same levels seen for the data from Cogan[7] that were used for distinguishing the event of YMB in eggs. From the data of Gast and Holt,[16] a model was developed that relates the exponential growth rate for SE in the vitelline membrane and temperature.

DISTRIBUTION OF TIME BEFORE YOLK MEMBRANE BREAKDOWN

YMB can be thought of as two events. First the yolk membrane is compromised, permitting SE cells access to the yolk material. Second, SE cells utilize the yolk nutrients. The likelihood of these events depends primarily on temperature. Considering the second event it is not difficult to imagine that the time at which rapid SE growth commences would depend on the location and the levels of SE contamination within the egg at the time the yolk membrane is compromised. The likelihood of SE cells using yolk nutrients, all else being equal, depends upon the number of SE cells in the egg. In addition, the location of the SE contamination (*Eac* or *Eaf*) would affect the time at which rapid growth begins.

Data to distinguish clearly the two YMB events are not available. Data from Cogan,[7] in which whole eggs were inoculated with different levels of SE in the albumen near the yolk, showed a slight dependency of the levels of SE on YMB. Data used to model the percentage of eggs that have experienced YMB, as a function of time and temperature, were provided by Humphrey.[8] The experimental protocol for the studies from which these data were generated was as follows:

(i) dry intact eggs were obtained from a local farm within two hours of lay;
(ii) intact un-inoculated eggs were stored at temperatures ranging from 12-37°C;
(iii) at selected intervals of days, 10 eggs were removed from storage and the contents were broken into a sterile container;
(iv) each egg was inoculated into the albumen next to the intact yolk with 500 cells (2.7 $\log_{10}$) of an overnight culture of a human clinical isolate of SE PT4 that was egg-associated;
(v) inoculated eggs were stored at 20°C for 4 days;
(vi) and SE were enumerated in each egg. The reported units of measurement were per egg, not per ml.

Point 4 implies that these data represent *Eac* eggs with the same number of SE cells. Thus, results from this study do not provide information about *Eaf* eggs or about the effect of SE numbers in contamination. These data have limitations for determining the probability distribution of YMB as a function of age and temperature of eggs. They reflect SE growth for approximately four days at 20°C, which could vary because the temperatures of the pre-inoculated eggs, while stored, were not 20°C. It is possible that YMB took place after

inoculation, within the 4 days of potential growth. The number of days stored at given temperatures affects the time of YMB. If eggs were contaminated, then the storage temperatures would particularly affect the duration of lag phase and subsequent SE growth. Because eggs were not contaminated until inoculation, these data may not provide a clear picture of the effects of time and temperature on the YMB event, and on subsequent growth for transovarian SE-contaminated eggs. Nonetheless, the data were used here to develop a model for determining the time of YMB for an *Eac* egg. To make some allowances for the likelihood of the second YMB event depending on the number of cells, which would be changing as a function of time if SE were growing within albumen, the probability function of YMB was assumed related to the level of contamination at the time of YMB. State of knowledge adjustment factors were used to reflect differences of distribution of times of YMB that may exist between *Eac* and *Eaf* eggs. Another state of knowledge adjustment was made to reflect differences of predicted probabilities of YMB that exist between these data and those of Cogan.[7]

To determine the distribution of time before YMB, it was necessary first to determine the criterion of YMB for an egg in the data from Humphrey.[8] The histogram in Figure E1 shows a tailing off of values at approximately 5 $\log_{10}$ cfu/egg, which is approximately 3.3 $\log_{10}$ cfu/ml, assuming an approximate 50 ml per egg. This represents an approximate relative growth of 2.3 $\log_{10}$ cfu/ml, or approximately 7 or 8 generations. The inoculated eggs were stored for 4 days. It can be reasonably assumed that SE cells would need to go through a period of acclimation or lag before beginning to grow, particularly because the inoculated cells were in stationary phase. Thus, the level of SE in eggs before YMB would be expected to be less than the level in the data from Cogan,[7] in which the contaminated eggs were stored for 8 days at 20 or 30ºC. Using the model developed above and assuming a ratio of mathematical lag to generation time of 5, for each egg that had inoculated levels of 25 or 250 SE cells, the expected amount of relative growth for an assumed 4-day storage period was calculated using the estimated value, μ, that was derived for that egg. The maximum of these expected values was approximately 2 $\log_{10}$ relative growth. Assuming an initial level of 2.7 $\log_{10}$, the above calculation implies an approximate maximum level of 4.7 $\log_{10}$ of SE for 4 days growth before YMB. Thus, for the data from Humphrey,[8] a cutoff value of 4.7 $\log_{10}$ for distinguishing YMB was used.

Using this level as a criterion for YMB, the cumulative distribution function (cdf) of the time of YMB as a function of a fixed temperature was determined. In reality, temperature could be changing. Therefore, what actually needed to be modeled was the probability of YMB at time t, the age of the egg in days, given a pathway of temperatures until t. More precisely, what was needed was the hazard function, $h(t, T)$ of YMB at time t. The necessary calculations involve accumulating conditional incremental probabilities that YMB will take place, given that it has not occurred at time t. Given the set of functions, $\{P(t, T)$, for all $T\}$, to calculate the probability for the path of temperatures, defined by $T(t)$, it was assumed that the hazard function does not depend upon the pathway, $T(s)$, for $s<t$. Let $h(t, T)$ be the hazard function of the cdf $P(t, T)$, that is,

$$h(t,T) = \frac{p(t,T)}{1 - P(t,T)} \tag{E7}$$

where $p(t, T)$ is the density function of t for a given T.

The above equation shows that given that YMB has not occurred up to time t, the probability of YMB in the increment of time at t is not dependent upon the previous temperature profile,

$T(s)$, for $s<t$. This assumption is most likely not true. The characteristics of the yolk membrane at any given time depend upon its history. The cells' abilities to migrate through the yolk membrane may depend upon their history. There are, however, no data available to develop a more realistic model. Thus, this assumption was one of necessity to make calculations. Given this assumption, the cdf of the time, t, of YMB, given a time t_0 for which YMB has not occurred, where $t_0 < t$, is

$$P(t,T(s), t_0 \le s \le t) = 1 - exp\left[\int_{t_0}^{t} h(s)ds\right] \qquad \text{(E8)}$$

so that $P(t_0,T(s), t_0 \le s \le t) = 0$.

Because YMB depends upon the number of SE cells in the egg, the hazard function is considered proportional to the number of cells at the time of YMB (see below). The objective of the analysis was to determine the hazard function so that Equation E8 may be applied to compute the probability of YMB at a given time. First, the derivation of the functional form of the cdf using the data from Humphrey[8] is described below.

$P(t, T)$ is the probability that growth from YMB begins, so that the rapid growth of SE cells due to yolk nutrients begins at time t. It was further assumed that this growth entails a lag, accounting for SE cells in the albumen to acclimate before utilizing nutrients for optimal growth. For these data, $P(0, T)$, which should not depend on T, need not be zero and would be interpreted as eggs that have unusually porous yolk membranes permitting SE cells to migrate to the yolk when the egg is laid. The parameter $P(0, T)$ was not used directly in determining the time of YMB for a given scenario. The influence of $P(0, T)$ is in determining the shapes of probability curves, expressed through the hazard functions. Equation E8 was used, where $t_0 = 1$ day, because it was assumed that the fraction of Ey, $\chi(Ey|T(s_0))$. This serves as the estimate of $P(1,T(s_0))$, where $T(s_0)$ is the temperature profile assumed for the study by Gast and Holt.[16] For a different temperature pathway, $T(s)$, the estimate of $\chi(Ey|T(s))$ was determined from

$$\chi(Ey\,|\,T(s)) = \chi(Ey\,|\,T(s_0))\frac{P(1,T(s))}{P(1,T(s_0))} \qquad \text{(E9)}$$

where $P(1,T(s))$ and $P(1,T(s_0))$ were determined from Equation E8 with $t_0 = 0$.

For estimating $P(t, T)$, using data from Humphrey,[8] the time represents egg age when inoculated. Because the measurements were made 4 days later, it is possible that YMB occurred within these 4 days. Based on the model for rapid growth in yolk, presented below, it is possible that in 2 days there would have been a 2 $\log_{10}$ increase of SE. There would be a lag of unspecified length, which would delay growth. To adjust for the possibility that lag could have taken place later than t, the independent variable $t + 2$ was used.

To determine the functional form of $P(t, T)$, the initial step was a graphical examination of the data. The data presented in Table E3 indicate that for a small t, $P(t, 20)$ is flat, and the projection of $P(t, T)$ to $P(0, T)$ is not zero. This would suggest functions such as

$$P(t,T) = 1 - exp\left[-exp(-a(T) + b(T)t^{c(T)})\right] \qquad \text{(E10)}$$

where $a(T)$, $b(T)$, and $c(T)$, are functions of temperature, or a Weibull distribution

$$P(t,T) = 1 - exp(-\alpha(T) - (t / \beta(T))^{\gamma(T)}) \quad \text{(E11)}$$

where $\alpha(T)$, $\beta(T)$, and $\gamma(T)$ are unknown parameters. For each temperature, maximum likelihood estimates (MLEs) were determined for four models: two-parameter extreme value and Weibull functions (where it is assumed that $c(T) = 1$ and $\alpha(T) = 0$) and three-parameter extreme value and Weibull functions. MLE (-2 ln(maximum likelihood) results are presented in Table E6.

TABLE E6 -2 LN(LIKELIHOOD) (IGNORING BINOMIAL CONSTANTS) AT MAXIMUM LIKELIHOOD ESTIMATES FOR DIFFERENT DISTRIBUTIONS FITTING FRACTION OF SAMPLES >4.7 LOG_{10} CFU/ EGG.

Temp (ºC)	Two-parameter Weibull	Three-parameter Weibull	Two-parameter Extreme Value	Three-parameter Extreme Value
12	155.56	155.54	156.48	155.62
16	158.62	158.33	159.28	158.94
20	176.98	168.32	171.82	168.52
23	57.32	57.32	57.85	57.45
24[a]	369.80	356.67	356.96	355.82
25	167.67	167.17	166.49	166.39
27	273.95	273.95	275.63	274.83
30	191.60	191.60	202.14	192.01
37	27.05	27.05	27.49	27.12
Sum	1578.57	1555.95	1574.14	1556.70

[a]One data point at t = 15 days, fraction = 0 from 9, excluded

We see from that for most temperatures the two-parameter models provide an adequate fit, even though, over the 9 temperatures examined, based on the omnibus chi-square test with 9 degrees of freedom, they do not provide adequate fits at significance levels less than 0.05 when compared to the three-parameter model. The two-parameter Weibull model does not provide adequate fits at 20 or 24°C, whereas, the two-parameter extreme value does not provide adequate fits at 20 or 30°C. Plots of the fitted curves and observed fractions for these temperatures are shown in Figure E4. The plots reveal the reasons for the lack of fit.

At the two lower temperatures, the lack of fit is due to the flatness of the curves for small and moderate values of t. This leads to a significant non-zero estimate of the probability at t = 0. At 30°C, the two-parameter extreme value distribution does not provide a good fit, compared to the other distributions for the diametrically opposed reason: the probability curves do not appear to be flat, but they increase rapidly with time forcing the projection at t = 0 to be near zero. Even so, the fitted two-parameter curve does not appear to provide a poor visual fit. Excluding the 20°C data set, the two-parameter extreme value distribution appears to provide adequate visual fits for all temperatures while permitting estimated non-zero probabilities at t = 0.

Using the two-parameter functions permits the use of computer programs to aid in determining a model. Logistic regressions (PC-SAS, release 8.0) using the log-log link, permits fitting the extreme value and Weibull functions with ln(t) as an independent variable. A step-wise selection procedure was performed, where the independent variables included temperature, time, ln(time), the squares and square roots of time and temperature, and the product of these terms. If the selected terms include one that is a function of the ln(t), then, essentially, a two-parameter Weibull function is chosen because the probability at t = 0 would necessarily be zero. Such was the observation when all data were included. When the highly influential data at 37ºC

were eliminated, a relatively simple model was selected which did not involve ln(t). This latter analysis points out the uncertainty regarding the inference concerning the probability at t = 0. That is, by excluding data from one temperature (37ºC), the functional form of the model was greatly affected. Because the ln(t) term is not selected when data at 37ºC are deleted, not using a two-parameter Weibull distribution for modeling YMB is justified.

Examination of the estimated parameters for the other models revealed a high degree of variability for those associated with the three-parameter models in addition to the (comparative) deviant results for 37°C. Table E7 shows the estimated parameters for the three-parameter functions, and the two-parameter extreme value functions.

TABLE E7 ESTIMATED PARAMETERS FOR THREE-PARAMETER FUNCTIONS, AND THE TWO-PARAMETER EXTREME VALUE FUNCTIONS.

Temp (°C)	No. Obs.	Three-parameter Weibull			Three-extreme Value			Two-extreme Value	
		Intrcpt. (alpha)	Location (beta)	Power (gamma)	Intrcpt. (alpha)	Slope (beta)	Power (gamma)	Intrcpt. (alpha)	Slope (beta)
12	328	0.0036	121.3	1.789	12.963	6.05	0.162	4.392	0.061
16	239	0.0216	95.2	1.928	12.105	6.28	0.140	3.645	0.051
20	318	0.0527	46.2	8.250	3.149	0.00	3.035	4.317	0.082
23	78	0.0000	31.7	2.836	10.201	3.20	0.339	4.729	0.164
24	300	0.2950	24.4	5.004	1.577	0.00	1.962	2.353	0.104
25	167	0.0462	22.6	2.652	3.877	0.36	0.767	3.235	0.145
27	256	0.0000	17.4	2.603	4.187	0.54	0.712	3.137	0.175
30	207	0.0000	13.3	2.565	50.567	44.31	0.051	2.927	0.207
37	50	0.0000	6.0	8.023	50.123	37.41	0.163	9.340	1.560

For the three-parameter Weibull, the location parameter β was log linear with temperature, but other parameters appeared variable, with no apparent function of temperature that could be used to fit the observed values. In particular, the intercept, α, for T = 24°C appeared deviant, as did results for $\gamma(T)$ at 20 and 37°C. The relationships of parameters for the three-parameter extreme value appeared, in this respect, even more variable. Consequently, the two-parameter extreme value function was left for consideration. With the exception of the estimated parameter values at 37°C, the estimates were linear, including nearly constant, with temperature. Transformations ln(a) and ln(ln(b) + k), where k is a constant, produced variables that were nearly linear with temperature. Fitting this model provided a simple but satisfactory model, even though for some temperatures the fit was not ideal. Figure E4 shows plots of ln(a) and ln(ln(b) + k) versus temperature where k = 3.5, determined from a nonlinear regression, as described below.

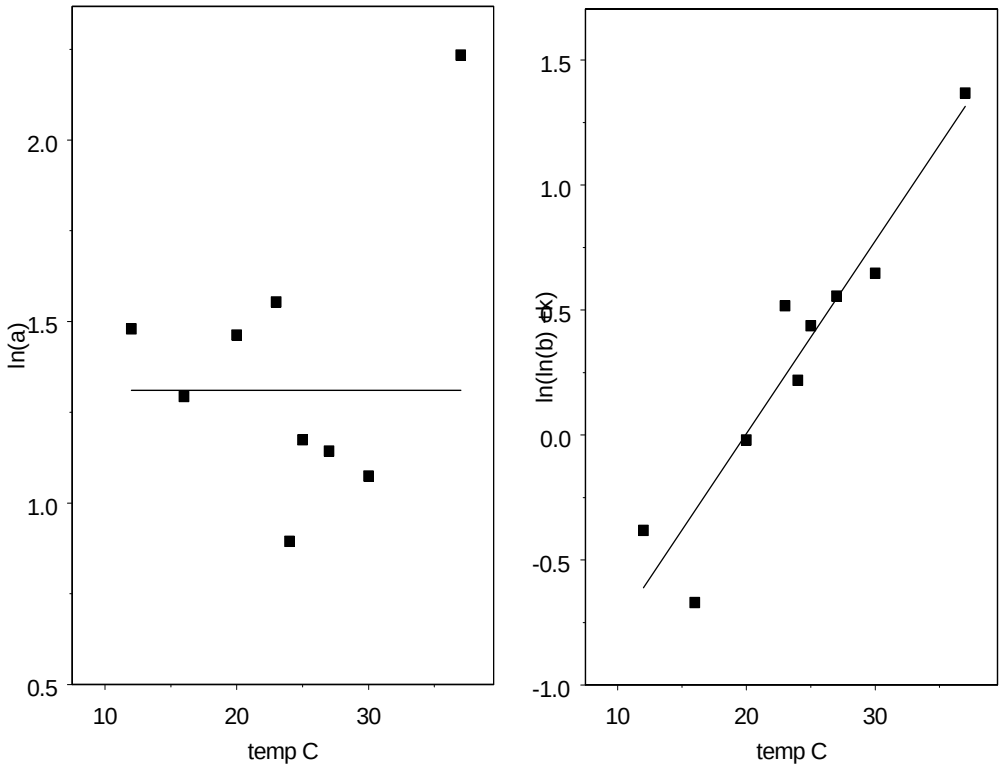


FIGURE E4 PLOT OF TRANSFORMED VALUES: LN(*A*) AND LN(LN(*B*) + 3.5) VERSUS TEMPERATURE, WHERE *A* AND *B* ARE ESTIMATES OF PARAMETERS OF EXTREME VALUE DISTRIBUTION, DEFINED IN EQUATION E10.

For determining the probability of YMB as a function of temperature and time, the extreme value function of Equation E10 is used and the values of the parameters are computed as

$$\begin{aligned} \ln(a(T)) &= d \\ \ln(b(T)) &= e^{f + gT} - k \end{aligned} \tag{E12}$$

where d, f, g, and k, are parameters, and T is temperature (°C).

TABLE E8 ESTIMATED VALUES OF PARAMETERS DEFINED IN EQUATION E9, WITH STANDARD ERRORS AND CORRELATION MATRIX.

Parameter	***d***	***f***	***g***	***k***
Estimates	1.3103	-1.5087	0.0751	3.4825
Standard Errors	0.1117	0.8829	0.0226	0.4299
d	1.0000	-0.0000	0.0000	-0.0504
f	-0.0000	1.0000	-0.9937	0.9718
g	0.0000	-0.9937	1.0000	-0.9452
k	-0.0504	0.9718	-0.9452	1.0000

Nonlinear, seemingly unrelated regression (SUR), weighted by the number of observations, was performed. The estimates and pair-wise mutual correlations are presented in Table E8. There are 6 degrees of freedom for the parameters *f*, *g*, and *k*. Observed data and fitted probability curves are shown in Figure E5.

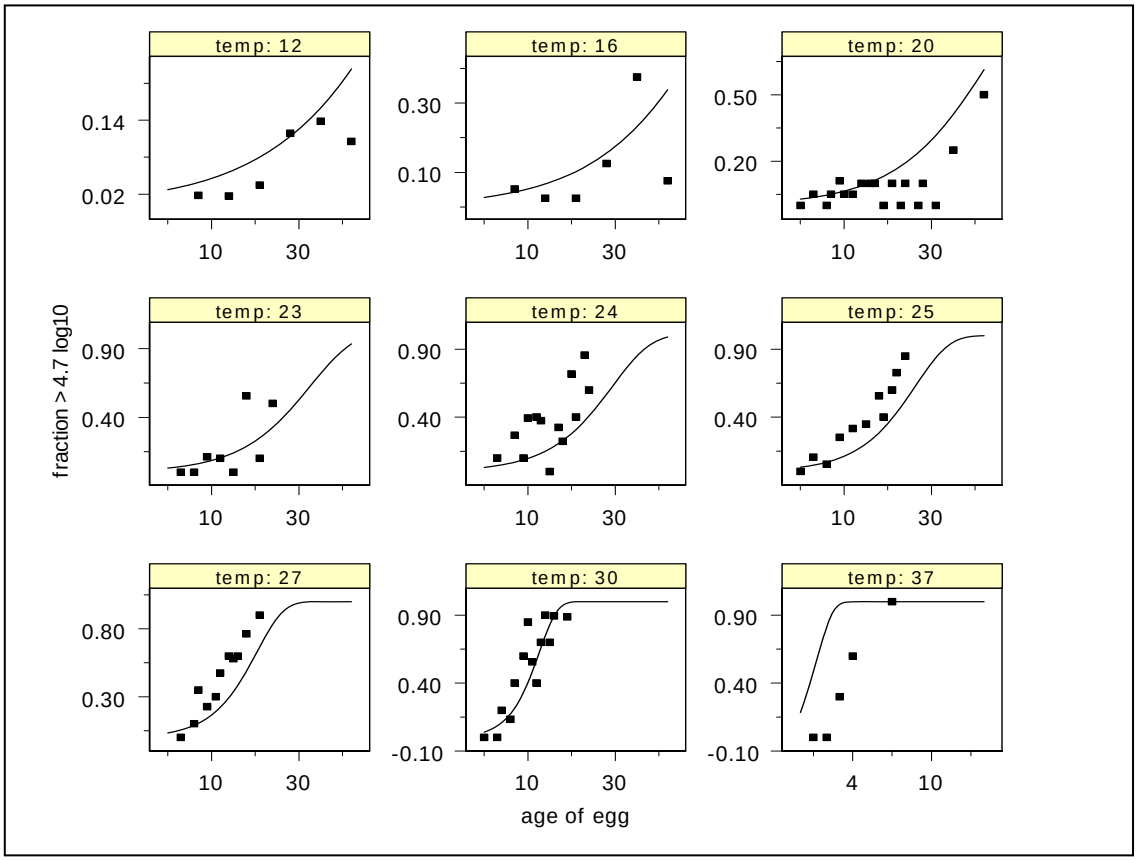


FIGURE E5 PLOT OF OBSERVED AND PREDICTED PERCENTAGES OF EGGS WITH GREATER THAN 4.7 LOG_{10}, DERIVED FROM DATA FROM HUMPHREY,[8] WHERE EACH EGG WAS INOCULATED WITH 500 CELLS AFTER BEING STORED AT DESIGNATED TEMPERATURE AND STORED 4 DAYS AFTER INOCULATION.

Difference between data of Cogan[7] and those of Humphrey[8]

To account for the discrepancies, and thus the large amount of uncertainty, that arose from the above comparisons between data of Cogan[7] and Humphrey,[8] an adjustment was made to $b(T)$. By

multiplying the parameter $b(T)$ in Equation E10 by Γ, where Γ is considered to be a state of knowledge parameter, taking the values of *1* and, Ω, where this last value is determined so that, at 25°C, the predicted fraction of 8-day-old eggs that would have undergone YMB is equal to that predicted from the data of Cogan[7] using results from inoculum levels of 25 and 250 SE cells. That is,

$$\Omega = \frac{\ln(-\ln(1-p)+a}{8b(25°C)} \tag{E13}$$

where: $a = a(T)$, constant for all T, and b(25°C) are determined from Equation E12; p is a random variable with expected value = 35/120, representing the 35 eggs that had more than 5.3 $\log_{10}$ cfu/mL from the 120 eggs that were inoculated with 25 or 250 cells.

When p = 35/120 and the other variables equal their expected values, Ω = 2.53. The stochastic uncertainty of p is determined assuming the number of eggs that showed YMB is distributed as a random variable with a binomial (or a normal) distribution.

Effect of inoculum level on probability of SE growth

The effect of the inoculum level on the probability of SE growth following YMB is also a concern. Results from a logistic regression were used to adjust the values of b in Equation E10. From the data of Cogan,[7] using the samples that were inoculated with 25, 250 and 2,500 cells and using these levels as the independent variable, a logistic regression for the probability of YMB using the log-log link was performed. The slope of this regression, corresponding to the value of b in Equation E9, was estimated to be 0.000319, with a standard error of 0.000106. The estimated probability an egg was contaminated with SE ranged from approximately 28% when there were no SE cells, i.e., the egg was not contaminated, to approximately 50% when there were 2,500 SE cells. Assuming the above model (Equation E12) holds for 500 SE cells, if L cells existed at a given time, the adjustment to $b(T)$ of Equation E10, and thus the hazard function, would be

$$b(L,T) = \frac{0.000319(L-500)}{8\Gamma} + b(T) \tag{E14}$$

where the original estimates apply for 500 SE cells. For L = 0, the value of $b(T)$ would be adjusted downward by 0.00789. If L = 50, the adjustment is 0.00710.

Growth of SE in* Eac *versus* Eaf *eggs

We did not identify data with which to estimate the distribution of time of YMB for *Eaf* eggs. Results from Humphrey et al.,[18] in which the authors compared increases in the number of SE cells in 2-week-old eggs after SE were inoculated in the albumen at various distances from the yolk, indicate a substantial effect of location of contamination (*Eaf* versus *Eac*) on the amount of SE growth. Figure E6 shows a graphical depiction of results from study.

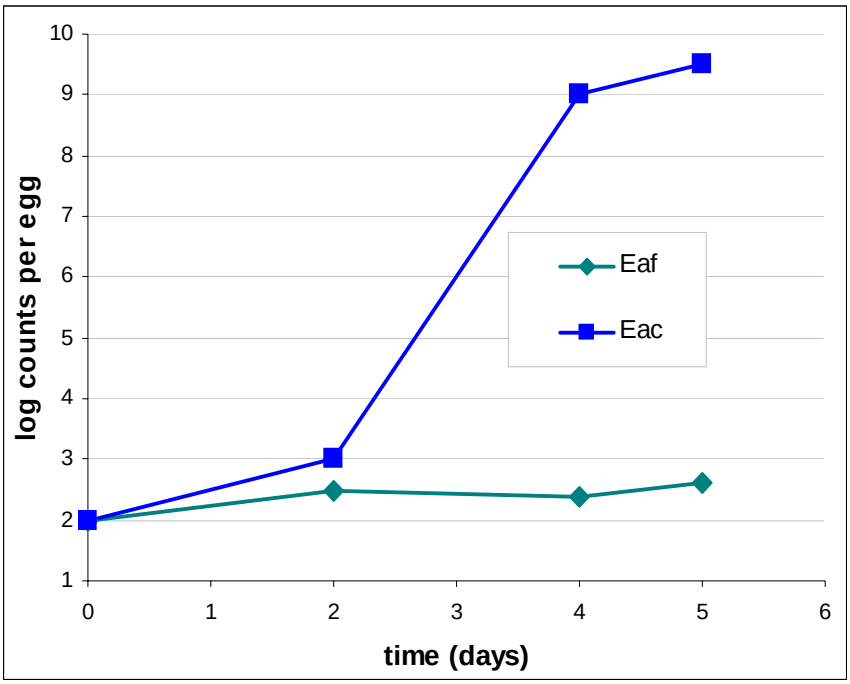


FIGURE E6 LOG_{10} OBSERVED COUNTS PER EGG VERSUS NUMBER OF DAYS OF STORAGE AFTER INOCULATIONS OF CA. 3 LOG_{10} SE CELLS. EGGS WERE TWO WEEKS OLD, AND STORED AT 20°C.[18]

Data are not available to develop estimates for modeling the relationships implied by Figure E6. The numbers of samples, the fraction of samples associated with SE growth, and the ranges of SE growth were not provided; nor were data available for modeling potential growth differences at multiple temperatures. Assuming that the number of samples and the inoculum levels were similar for the *Eac* and *Eaf* eggs, Figure E6 indicates the potential for substantial SE growth in *Eac* eggs, and at the same time, little SE growth potential for *Eaf* eggs. The cause of the growth observed in the *Eac* eggs is likely YMB. Thus, Figure E6 suggests YMB occurred for *Eac* eggs and not for *Eaf* eggs. To reflect this difference in growth potential a state of knowledge parameter varying in value from 0.25 to 1 was created and multiplied by *b* of Equation E10 to estimate the probability distribution of the times of YMB for *Eaf* eggs. The same factor was applied for *Es* and *Ep* eggs.

SE GROWTH IN THE PRESENCE OF YOLK NUTRIENTS

YMB represents the time when SE growth kinetics switch from those operative in albumen to those operative in the presence of yolk nutrients. Following YMB, a lag phase presumably would occur before SE growth begins. The situation within the egg immediately after YMB may be described by considering instantaneous probabilities of SE cell division from either albumen without presence of yolk nutrients or yolk nutrients. Imagine four compartments for SE cells at any time: (i) original cells still in lag phase with respect to albumen and yolk; (ii) original cells in exponential phase with respect to albumen and in lag phase with respect to yolk; (iii) original cells in lag phase with respect to albumen and exponential phase with respect to yolk; and (iv)

cells in exponential phase with respect to albumen and yolk. Offspring cells would be in the latter compartment.

Thus, if $N_{ij}(t)$, i , j = 1, 2, is the number of cells at time t, the two phases for the cells with respect to albumen and yolk (i and j, respectively), where an index value of 1 represents lag phase and 2 represents exponential phase, the system of equations describing these dynamics is

$$\begin{pmatrix} dN_{11}(t)/dt \\ dN_{12}(t)/dt \\ dN_{21}(t)/dt \\ dN_{22}(t)/dt \end{pmatrix} = \begin{pmatrix} -(\lambda_A + \lambda_Y - \lambda_A\lambda_Y) & 0 & 0 & 0 \\ \lambda_Y & -\mu_Y & 0 & 0 \\ \lambda_A & 0 & -\mu_A & 0 \\ \lambda_A\lambda_Y & \lambda_Y & \lambda_A & \mu_A + \mu_Y \end{pmatrix} \begin{pmatrix} N_{11}(t) \\ N_{12}(t) \\ N_{21}(t) \\ N_{22}(t) \end{pmatrix}$$

where λ and μ represent parameters that determine lag phase and exponential growth rates, respectively, and the subscripts A and Y represent the growth medium of albumen and yolk, respectively. When YMB begins, t_0, it is assumed that $N_{11}(t_0)$ and $N_{21}(t_0)$ are known and that the other quantities are zero. The parameters are a function of time due to changing temperatures within the egg. The values of the parameters associated with yolk nutrients are larger than parameters associated with albumen. In effect, once the yolk nutrients become available, the growth expected to occur depends on cell dynamics with respect to yolk. Thus, for practical purposes, the dynamics with respect to albumen can be ignored without creating a significant bias in estimating the number of cells at any given time. Consequently, when YMB occurs, it is assumed that all SE cells follow growth kinetics associated with the yolk nutrients.

RAPID RELATIVE GROWTH OF SE THAT HAVE ACCESS TO YOLK NUTRIENTS

Following YMB, the increased concentration of available nutrients raises the potential for exponential growth of SE within eggs. Maximum growth of SE is expected during this time. Consequently, an estimation of growth rate for SE cells in the presence of yolk nutrients constitutes a critical section in the modeling of SE growth in eggs. To estimate growth curve values for SE cells growing exponentially in the presence of yolk nutrients, data from five studies in which SE were inoculated into egg yolks were examined.[13,9,14,16,11] Generally, SE growth curves were found to be log linear (ln of the measured level versus time) to some point after which the rate of growth lessened to levels indicative of stationary phase. Because the maximum temperature examined in these five studies was 37°C, the results could not be used to model reliably growth of SE over the full range of internal egg temperatures. (For example, egg temperature at lay is approximately 41.1ºC, the body temperature of the hen.) To bridge this information gap, data from a study of SE growth in broth solution (pH 7.2) at temperatures ranging from 7 to 42°C[10] were used. The mean exponential growth rates predicted in this study were not significantly different from those observed in yolk studies. Using the results from these combined studies, a model for predicting exponential growth rates of SE for temperatures below 42°C was derived. Graphical results listed below by temperature and study were examined. Each of the studies included experiments in which SE were inoculated into yolk material. In addition, the study conducted by Gast and Holt[16] included experiments in which yolk surfaces (vitelline membranes) were inoculated. Each of these experiments is discussed below.

Saeed et al.[13] inoculated yolks of 65-70 g eggs with 20 cfu/egg of SE and measured SE levels in eggs. To determine the initial levels of SE, it was assumed that the yolks were approximately 20 g. Thus, initial SE levels were approximately 1/mL or 0 $\log_{10}$/ml. Bradshaw et al.[9] describe two experiments performed at 37°C, one for eggs that were labeled as coming from normal hens, and one from eggs laid by hens whose sera reacted with Group D *Salmonella* antigens.

Schoeni et al.[14] presented graphs of SE growth curves at 4°C. For the lower density initial level (2 $\log_{10}$ cfu/g), there was a small amount of growth over a 7-day period to a level of approximately 2.7 $\log_{10}$ cfu/g. For the higher density initial level (4 $\log_{10}$ cfu/g), there was a decrease to approximately 3.6 $\log_{10}$ cfu/g. These results are ambiguous, particularly in light of the results obtained by Bradshaw[9] indicating little or no growth at 7°C. Because growth of SE at 4°C is expected to be negligible, results from experiments performed at 4°C were not used.

In the study by Gast and Holt,[16] the initial levels of SE inoculum were 15 and 150 cfu (densities not given). A later paper reported an inoculated level of 100 cfu as 8 cfu/ml.[11] This implies a volume of approximately 12 ml. If so, 15 cfu and 150 cfu translate to initial SE levels in the yolk material of 1.2 and 12 cfu/ml, respectively. For experiments where inoculations were on the yolk surface and measurements were made for the whole egg, an egg volume of 50 ml was assumed, which translates to initial levels of -0.5 and 0.5 $\log_{10}$ for the 15 and 150 cfu inoculation levels, respectively. Tables E9 and E10 give the assumed levels ($\log_{10}$ cfu/g or mL) versus times for those curves indicating growth.

TABLE E9 DATA USED TO DETERMINE GROWTH CURVE FOR SE IN YOLK. ENTRIES ARE $\log_{10}$/ML.

	Study												
	Saeed et al.[13]			Schoeni et al.[14]				Gast and Holt[16]				Gast and Holt[11]	
Time (d)	Temp (°C)			Temp (°C)				Temp (°C)				Temp (°C)	
	23	10		25		10		17.5		25		15	25
0	0.0[a]	2	4	2	4	0[b]	1[b]	0[b]	1[b]	0[b]	1[b]	0.9	0.9
1	6	2	4	6.2	8.1	1.7	2	3.2	4.2	6.5	7.2	1.2[c]	4.4[c]
												3.9	8.5
2	.	.	.	.	.	2.3	2.5	6.1	7	8.5	8.8	7	
3	8.2	3.6	5.6	8.8	9	3	3.1	8	8	9	9	7.5	
5	9.2	4.8	7	9	9.2	.	.	.	.	.	.		
7	8.8.	5.8	5.8	9	8.8	.	.	.	.	.	.		

[a]Estimated from reported 20 cfu/egg inoculum to be 0.0 $\log_{10}$/ml.
[b]Estimated from reported 15 and 150 cfu inoculum to be 0.079 $\log_{10}$/mL and 1.079 $\log_{10}$/ml.
[C]Times are at 6 hours. Approximately 55% of the sampled eggs were reported positive.

TABLE E10 DATA DERIVED FROM BRADSHAW ET AL.[9]

Temp (°C)											
15.5	Time (d)	0	1	1.5	2	2.5	4	5	6	7	8
	SE level	0	2	2.5	4.1	4.5	7.7	7.7	7.9	8	9
37 (seronegative hens)	Time (h)	0		4		8		12		16	24
	SE level	0		2.5		6.5		8		8.6	8.3
37 (seropositive hens)	Time (h)	0	4		8		12	20		24	48
	SE level	-0.3	2.3		4.7		6.5	6.9		7.2	8.0

For the no growth curve at 7°C, three data points were used: 0, 3, and 7 days. For two experiments at 10°C,[14] no growth was indicated for the first day. Thus, because lag times were not measured from these data, the values at time = 0 (t_0) were deleted and the growth curves for time ≥1 day were used.

For a given experiment, at a given temperature, a logistic growth model,

$$\frac{dS(t)}{dt} = \mu(1-\frac{S(t)}{M}) \qquad \text{(E15)}$$

was used, where $S(t)$ is the number of cells at time t; μ is the exponential growth rate, assumed constant; and M is the maximum population density at stationary levels.

The solution to the above equation is written[17] as

$$\ln(S(t)) = n_0 + \mu t - \ln\left[1+(e^{\mu t}-1)/e^{m-n_0}\right] + \varepsilon \qquad \text{(E16)}$$

where m is the natural log of the maximum level, M; s_0 is the natural log of the initial level; and ε is the residual error with an expected value equal to 0 and variance equal to σ^2.

TABLE E11 GROWTH OF SE ON YOLK SURFACE (VITELLINE MEMBRANE) BASED ON DATA FROM GAST AND HOLT.[11] ENTRIES ARE LOG_{10} CFU/ML.

Temp (°C)	Time (d)			
	0	1	2	3
17.5	0.5	1.9	3.6	5.4
25	-0.5	3.1	4.2	5.0
25	0.5	3.8	6.1	6.0

The data used for estimating the parameters are listed in Tables E7 a, b, c. Nonlinear regressions for Equation E16, estimating μ, m, and n_0, were performed for each experiment (PC-SAS, release 8.0). For temperatures ≤10°C, the logistic term on the right side of Equation E16 was deleted because these growth curves did not reach stationary phase. The results from this

regression show (Table E11) a relationship between temperature and maximum growth levels of SE.

TABLE E12 NONLINEAR REGRESSIONS OF EQUATION E16.

Study	Temp (°C)	Seropositive Hens	Yolk Surface	Exponential Growth Rate (day^{-1})	Growth Rate Square Root	Max. Level ln(cfu/g or ml)
Bradshaw et al.[9]	7.0	No	No	0.00	0.01	.
Schoeni et al.[14]	10.0	No	No	2.40	1.55	.
Schoeni et al.[14]	10.0	No	No	1.16	1.08	.
Gast and Holt[16]	10.0	No	No	3.13	1.77	.
Gast and Holt[16]	10.0	No	No	2.39	1.55	.
Gast and Holt[11]	15.0	No	No	9.79	3.13	17.06
Bradshaw et al.[9]	15.5	No	No	5.63	2.37	18.74
Gast and Holt[16]	17.5	No	No	9.01	3.00	18.43
Gast and Holt[16]	17.5	No	No	9.02	3.00	18.34
Gast and Holt[16]	17.5	No	Yes	5.24	2.29	13.22
Saeed and Koons[13]	23.0	No	No	18.01	4.24	20.11
Schoeni et al.[14]	25.0	No	No	13.41	3.66	20.57
Schoeni et al.[14]	25.0	No	No	13.30	3.65	20.72
Gast and Holt[16]	25.0	No	No	19.07	4.37	20.15
Gast and Holt[16]	25.0	No	No	18.34	4.28	20.49
Gast and Holt[11]	25.0	No	No	46.30	6.80	19.57
Gast and Holt[16]	25.0	No	Yes	11.87	3.45	10.59
Gast and Holt[16]	25.0	No	Yes	11.05	3.32	13.93
Bradshaw et al.[9]	37.0	No	No	59.33	7.70	19.12
Bradshaw et al.[9]	37.0	Yes	No	42.14	6.49	16.78

[a]Statistical outlier, based on a studentized residual of 4.8 (excluding the data point) obtained from a linear regression of data in the table, with the square root of the exponential growth rate as the dependent variable, and temperature as the independent variable. This data point was deleted from the following analysis.

Although similar results showing a temperature growth relationship have been shown by Marks et al.,[19] it is possible that the relationship does not reflect an inherent inability of cells to reach the high(er) maximum levels at lower temperatures. Instead, the relationship may reflect the inability of older cells (time since inoculation) to grow to the high(er) maximum levels observed for higher temperatures. It is also possible that at lower temperatures, the ability of cells to utilize nutrients is decreased, regardless of age, and thus cells were more likely to reach stationary phase at lower levels. It is not clear why the decreasing relationship exists. For modeling growth, we did not assume the maximum levels were temperature-dependent, but rather assigned a constant value for *m*.

Table E13 presents SE generation times and the derived exponential growth rates from data of Fehlhaber and Kruger.[10] From experiments conducted at 7°C, the authors report a coefficient of variation in excess of 2,000%, and that generation times "vary considerably between long positive and long negative periods." We assumed the phrase "negative periods" refers to long periods for which no SE growth was observed. The authors conclude their observation "means that below 7°C no growth of *Salmonella* Enteritidis can be observed." However, the possibility that growth could occur at temperatures below 7°C cannot be dismissed. Because of the large amount of uncertainty associated with this observation, the data point was deleted in the

following analysis. The resulting secondary model of growth rate and temperature predicts a minimum growth temperature of 4.5°C.

TABLE E13 MEAN GENERATION TIMES AND DERIVED EXPONENTIAL GROWTH RATES FROM FEHLHABER AND KRUGER.[10] MEAN RESULTS BASED ON 45 SE STRAINS.

Temp (ºC)	Generation Time (min)	Exponential Growth Rate (day^{-1})
7	747.00	1.34
9	595.00	1.68
12	349.00	2.86
17	129.00	7.74
22	65.00	15.36
27	38.10	26.20
32	24.70	40.41
37	20.40	48.93
42	24.00	41.59

These data were used to represent exponential growth rates that could be seen in the yolk of contaminated eggs. Figure E9 presents plots of the fitted square root curve for exponential growth rates (Equation E17) of the data in Table E3; the square root of the exponential growth rates (Table E13); and the fitted line for the data of exponential growth in yolk (Table E12). Because the fitted curves are close to one another, inclusion of data from Table E13 was judged not to introduce a significant bias.

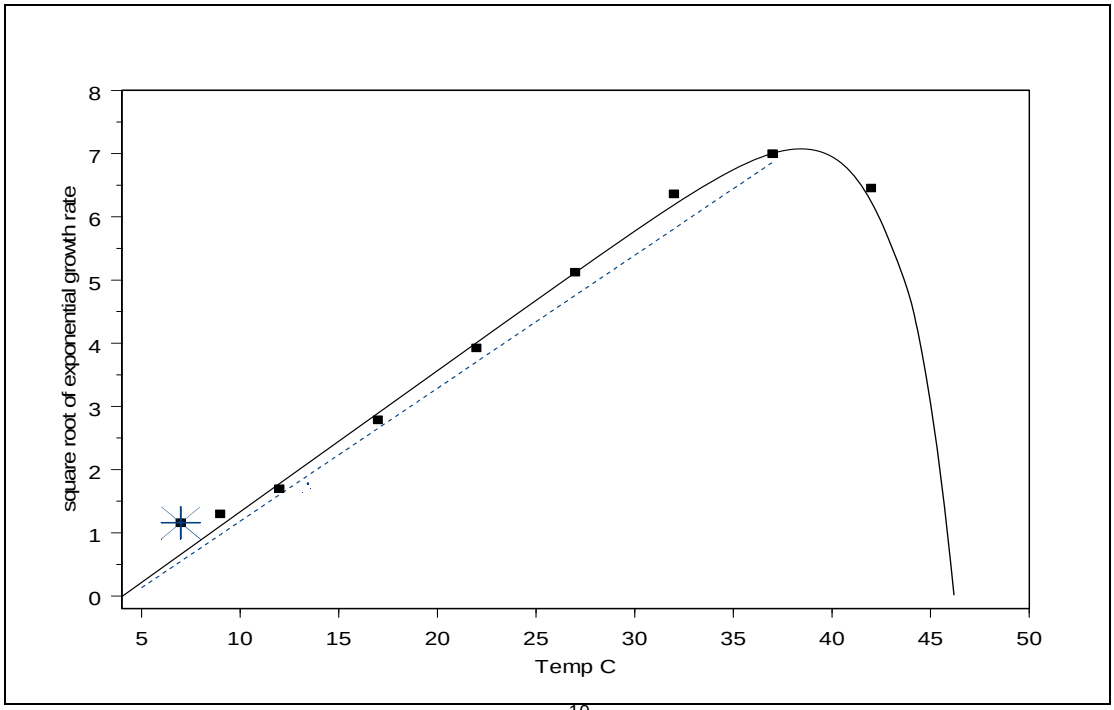


FIGURE E7 PLOT OF DATA IN TABLE E13,[10] THE FITTED SQUARE ROOT EXPONENTIAL GROWTH RATE CURVES FOR SE IN BROTH (THE SOLID LINE), AND THE ESTIMATED EXPONENTIAL GROWTH RATES FOR SE IN YOLK FOR DATA IN TABLE E8 (THE DOTTED LINE). DATA POINTS ARE FROM TABLE E13. DATA POINT AT 7°C NOT USED IN ANALYSIS.

To determine the relationship between the exponential relative growth rate, μ, and temperature, the square root model for the exponential growth rate[20] was used,

$$\mu^{1/2} = [(1+w\delta_w)(1+v\delta_v)]^{1/2}(e + fT + g\delta_g)(1- e^{b\,(T-T_{max})}) + \eta \qquad \text{(E17)}$$

where the variables are: T, the temperature (ºC); δ_w, an indicator variable for seropositive hens (δ_w = 1, if the egg is from a seropositive hen, otherwise δ_w = 0); δ_v, an indicator variable for vitelline membrane (δ_v = 1 if the growth curve is for the vitelline membrane, otherwise δ_v = 0); δ_g, an indicator variable regarding data source (δ_g = 1 if the observation is from Fehlhaber and Kruger,[10] otherwise δ_v = 0); the parameters for which values are to be estimated: e, f, g, b, T_{max}, w and v are fixed parameters; and η is a random variable with expected value equal to 0 and variance equal to ς^2, associated with the experiments or study.

By examining the results in Table E12 , comparing the estimated values of *:* for yolk-contaminated and yolk surface (vitelline membrane)-contaminated eggs from the same study,[16] it can be seen that the implied assumption of the above equation, namely, the exponential growth rates of SE on the vitelline membrane is a factor of those of SE in the yolk, seems reasonable. The estimates of μ for the yolk-contaminated eggs are nearly twice as high as the corresponding estimates for the yolk surface-contaminated eggs. The results appear to cluster by study and temperature. To account for this, seven clusters were defined by study and temperature, where all of the experiments by Bradshaw et al.[9] and by Saeed et al.[13] were placed in one cluster, with the exception of the result at 7°C. Data from Fehlhaber and Kruger[10] were assigned to the eighth cluster. A non-linear mixed effects model (PC-SAS, release 8.0) was used to estimate the parameters of Equation E17, with cluster as the subject variable. The parameter g was not statistically significant in the various models that were fit. Variations to the model of Equations E17 were tried, such as assuming that f was a function of δ_g or assuming $(1+g\delta_g)$ as a factor of μ. In all cases, g was not statistically significantly different from 0 (P-values near 0.7-0.8). Thus, the final model assumed g was zero.

Figure E8a presents plots of the within cluster residuals versus the within cluster predicted values, assuming within cluster homoscedastic variances (equal residual within cluster variances). It appears that for the lowest predicted values the variability of the residuals is larger. Assuming a model in which the standard deviation is proportional to a power of the predicted value, the estimated power was about -1, and was statistically significant at the 0.01 level. With this (homoscedastic variance) model, variability of the within cluster residuals is larger for small predictions and slightly smaller for large predictions (Figure E8b) than for the homoscedastic variance model. Figures E9 a and b present plots of the predicted and observed square roots of the exponential growth rates versus temperature using the estimated parameter values for the model of Equation E17, assuming homoscedastic and heteroscedastic variances, respectively, for the cases: growth in interior of yolk and in vitelline membrane, and eggs from and not from seropositive hens. The differences between the two figures are slight. For the homoscedastic model, the maximum predicted square root of the exponential growth rate is 7.10 at about 38.9ºC, while for the heteroscedastic model, the maximum predicted value is 7.09 at about 38.3ºC; the minimum temperature for which the predicted value is greater than zero is 4.53ºC for the homoscedastic model with standard error of 0.721, while for the heteroscedastic model the minimum temperature was estimated to be 5.43ºC, with standard error of 0.788 ºC. At 20ºC, the homoscedastic model predicts an exponential growth rate (transformed to $\log_{10}$ units per hour) in

the yolk (*Ey* contamination) of 5.11 (with standard error of 0.415), and, on the vitelline membrane (*Ev* contamination) of 3.14 (0.360). For the heteroscedastic model, the corresponding estimates are for *Ey*, 5.37 (0.454); and for *Ev*, 3.40 (0.367); thus, the estimates of the exponential growth rates are slightly higher for the latter model. The latter model was used here.

Tables E14 a and b present the estimates of the parameter values of Equation E17 for the homoscedastic and heteroscedastic models.

TABLE E14A ESTIMATED VALUES FOR PARAMETERS OF HOMOSCEDASTIC VARIANCE MODEL FOR PREDICTING EXPONENTIAL GROWTH IN YOLK OR YOLK SURFACE CONTAMINATED EGGS. ENTRIES BELOW THE STANDARD ERRORS ARE ELEMENTS OF THE CORRELATION MATRIX.

	e	***f***	***B***	T_{max}	***v***	***w***
Estimate	-1.0063	0.2219	0.4007	45.5733	-0.3864	-0.3030
Standard Error	0.1771	0.0065	0.1417	1.1838	0.0392	0.0426
e	1.0000	-0.6273	0.3388	-0.2626	0.1385	0.0462
f	-0.6273	1.0000	-0.6456	0.5685	0.0699	-0.0156
b	0.3388	-0.6456	1.0000	-0.9852	-0.0683	0.0891
T_{max}	-0.2626	0.5685	-0.9852	1.0000	0.0713	-0.0886
v	0.1385	0.0699	-0.0683	0.0713	1.0000	0.0043
w	0.0462	-0.0156	0.0891	-0.0886	0.0043	1.0000

TABLE E14B ESTIMATED VALUES FOR PARAMETERS OF HETEROSCEDASTIC VARIANCE MODEL FOR PREDICTING EXPONENTIAL GROWTH IN YOLK OR YOLK SURFACE CONTAMINATED EGGS. ENTRIES BELOW THE STANDARD ERRORS ARE ELEMENTS OF THE CORRELATION MATRIX.

	e	***f***	***B***	T_{max}	***v***	v_{pos}
Estimate	-1.3136	0.2418	0.2475	47.2896	-0.3674	-0.3137
Standard Error	0.2301	0.0103	0.0385	0.6384	0.0252	0.0139
e	1.0000	-0.7703	0.6851	-0.5720	0.0954	0.1680
f	-0.7703	1.0000	-0.9039	0.8430	0.2107	0.1120
b	0.6851	-0.9039	1.0000	-0.9807	-0.1939	-0.0421
T_{max}	-0.5720	0.8430	-0.9807	1.0000	0.2223	0.0692
v	0.0954	0.2107	-0.1939	0.2223	1.0000	0.1839
v_{pos}	0.1680	0.1120	-0.0421	0.0692	0.1839	1.0000

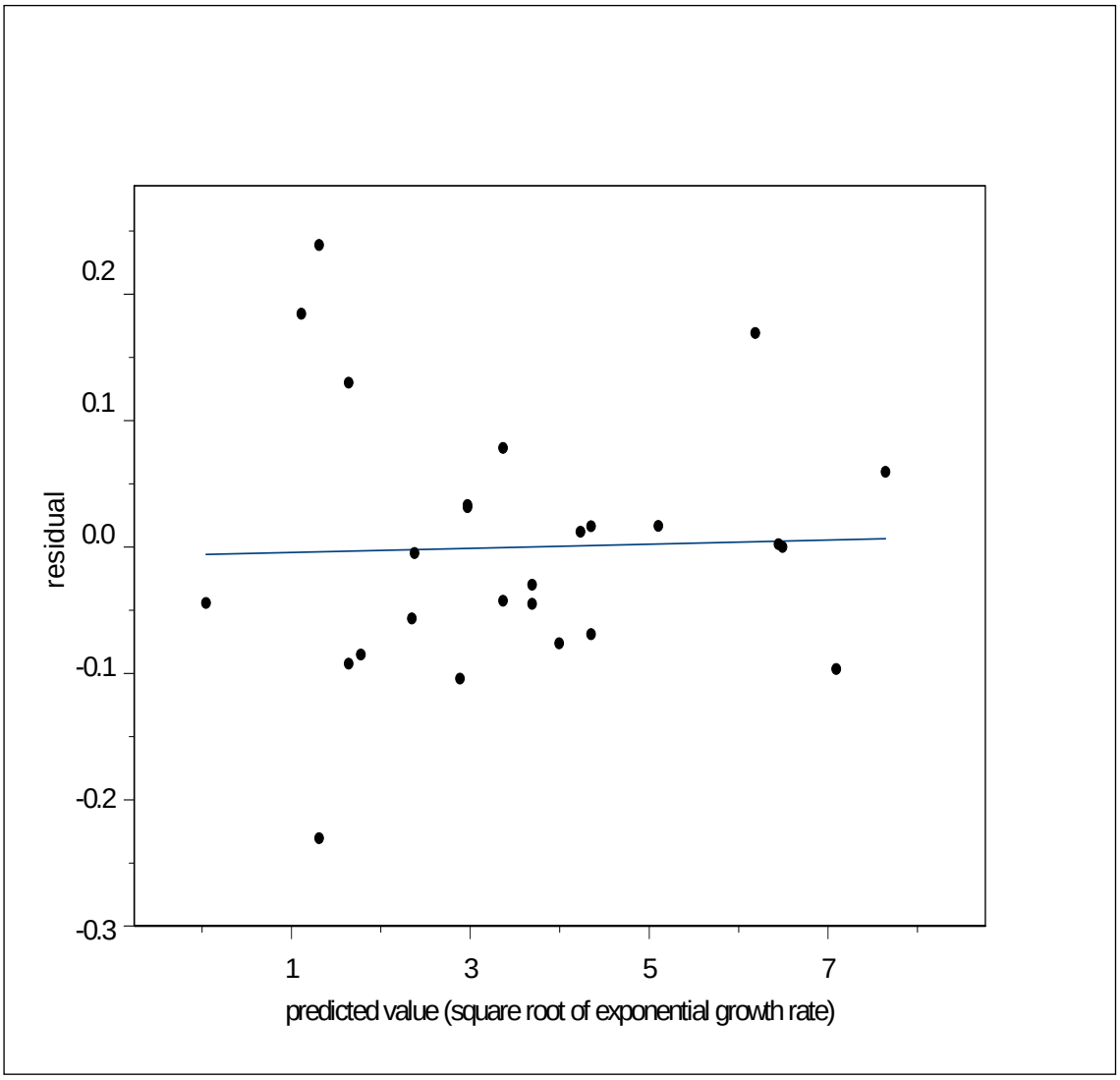


FIGURE E8A PLOT OF WITHIN CLUSTER RESIDUALS VERSUS PREDICTED VALUES FOR MODEL OF EQUATION E17, ASSUMING HOMOSCEDASTIC VARIANCE.

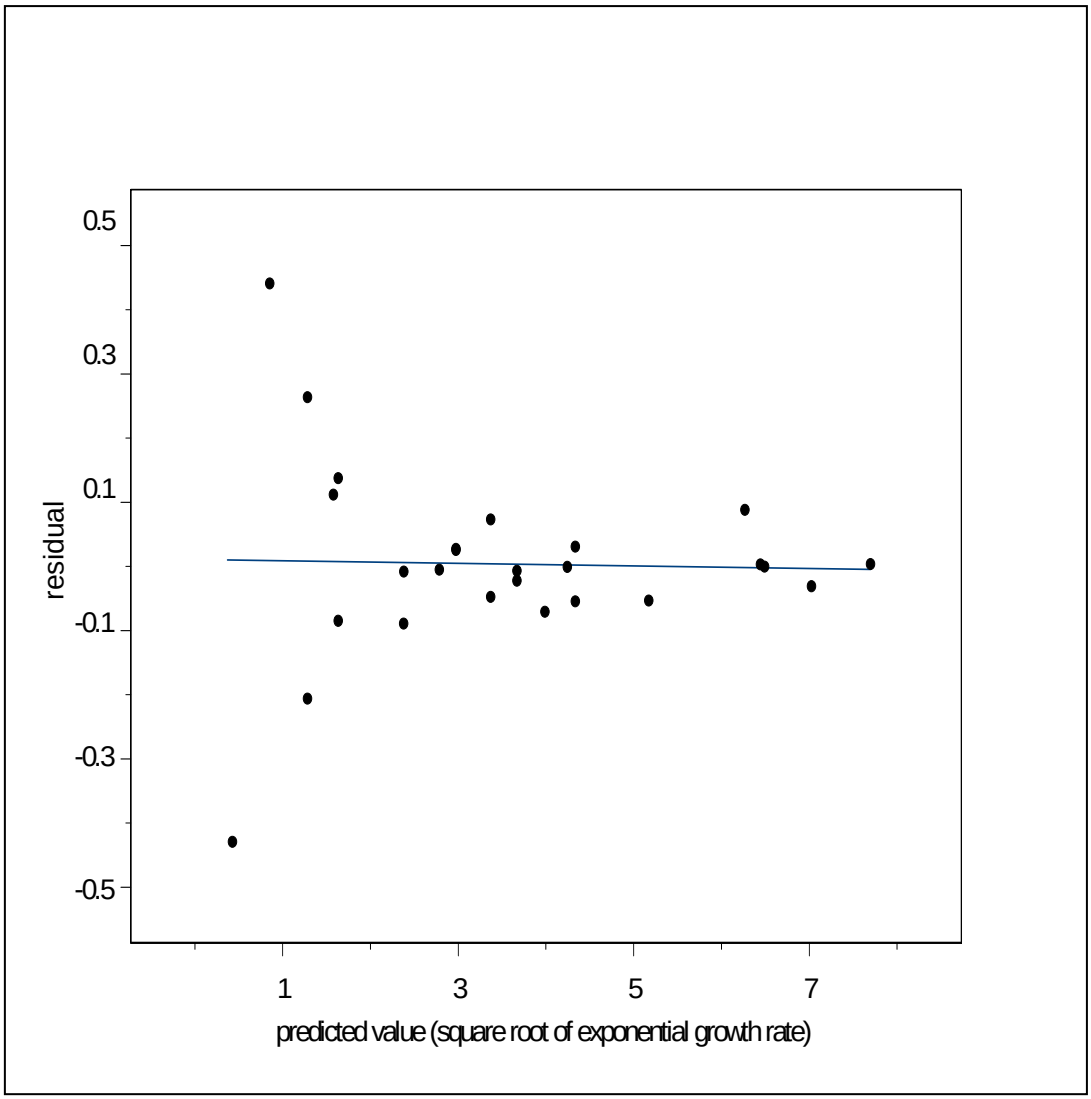


FIGURE E8B PLOT OF WITHIN CLUSTER RESIDUALS VERSUS PREDICTED VALUES FOR MODEL OF EQUATION E17, WHEN STANDARD DEVIATION ASSUMED PROPORTIONAL TO POWER OF PREDICTED VALUE (HETEROSCEDASTIC VARIANCE).

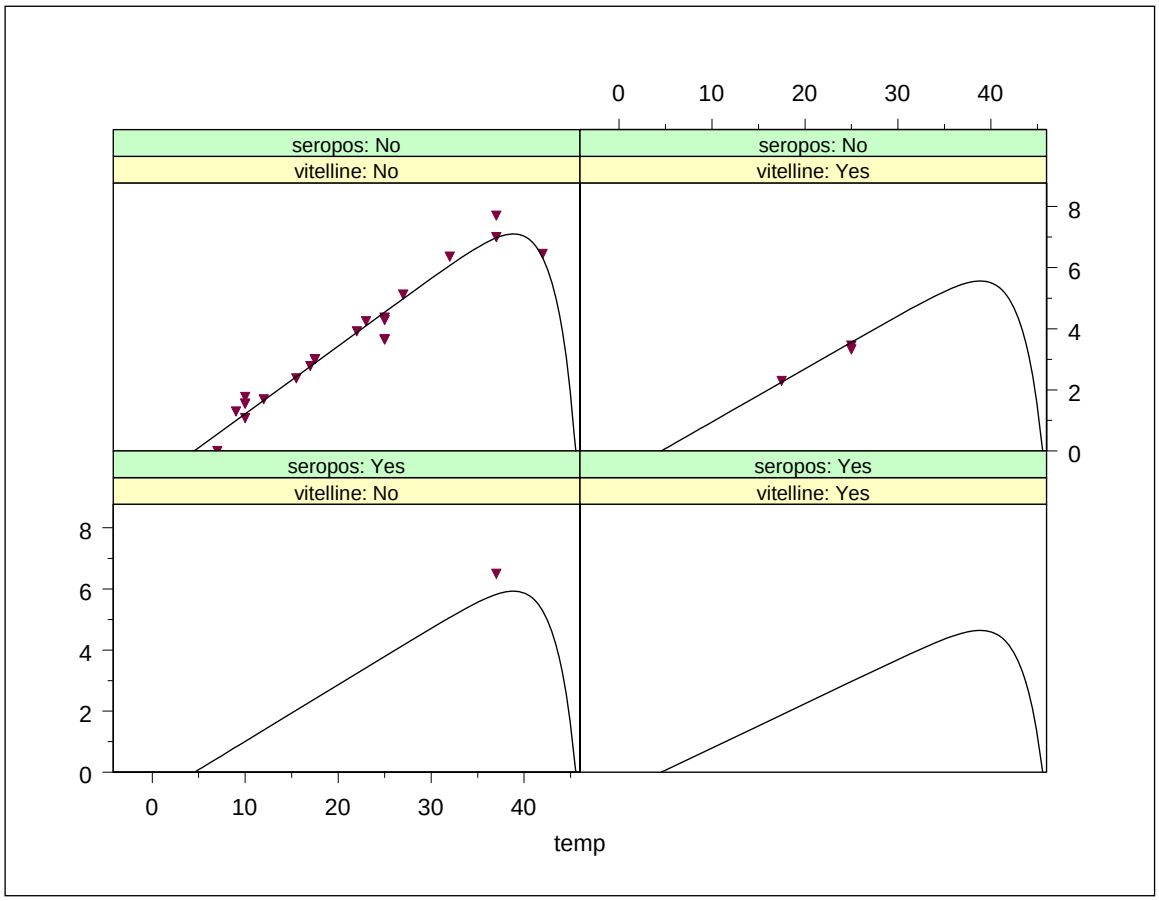


FIGURE E9A PLOT OF PREDICTED AND OBSERVED VALUES OF THE SQUARE ROOT OF EXPONENTIAL GROWTH RATES VERSUS TEMPERATURE, ASSUMING HOMOSCEDASTIC VARIANCE STRUCTURE, FOR THE CASES: GROWTH IN INTERIOR OF YOLK AND IN VITELLINE MEMBRANE, AND EGGS FROM AND NOT FROM SERO-POSITIVE HENS.

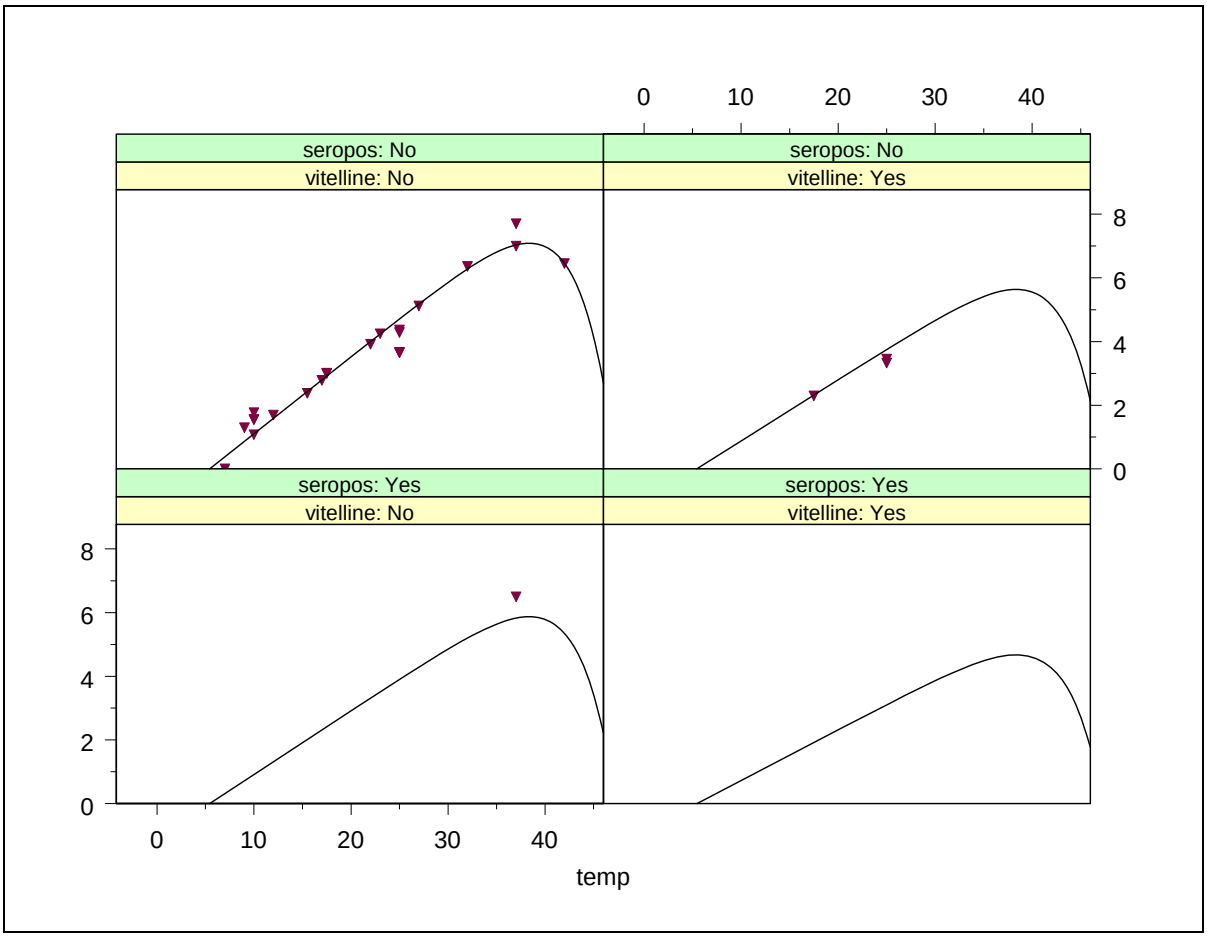


FIGURE E9B PLOT OF PREDICTED AND OBSERVED VALUES OF THE SQUARE ROOT OF EXPONENTIAL GROWTH RATES VERSUS TEMPERATURE, ASSUMING HOMOSCEDASTIC VARIANCE STRUCTURE, FOR THE CASES: GROWTH IN INTERIOR OF YOLK AND IN VITELLINE MEMBRANE, AND EGGS FROM AND NOT FROM SERO-POSITIVE HENS.

Incidence, Magnitude and Duration of Immune Response to SE

SE growth is likely hindered by the hen's immune response. Exposure to foreign molecules, such as SE, may produce an immune response within the hen. The response may include the production of antibodies, an important class of which is glycoproteins termed immunoglobulins. Hens supply their eggs with maternal antibodies that may provide protection against SE infection.[21;21-23] Up to ca. 25 mg/ml immunoglobulin G (IgG) have been detected in yolk, and egg albumen is known to contain IgM and IgA.[24] Yet the incidence and quantity of maternal antibodies within eggs depends on the magnitude of the maternal immune response, which in turn depends on dose and route of SE infection, frequency of re-infection, molting status, hen age, immune response, and genotype of both hen and infecting SE strain. Data available to address this issue are generally grouped into the three types of experiments, discussed below.

Artificially exposed hens

Earlier studies have shown that typically >90% of hens inoculated with $10^8$to 10^9 cfu/egg of SE exhibited peak serum antibody (IgG) titers between 7 and 14 days post inoculation.[11;18;25] Yolk antibodies had peak range of 10 to 28 days post-inoculation.[26-28] Thus, while high antibody titers can be acquired in yolk, their peak levels may occur as much as two weeks later than those in serum.[27;29;30] In addition, a strong dose-response relationship appears to exist between SE inoculum level and immune response. Hens orally inoculated with 10^3, 10^6, 10^8 cfu SE yielded peak serum IgG antibodies of OD_{405} 0.15, 0.4, and 0.84, respectively.[18] Likewise, in hens orally inoculated with 7.5 x 10^3, 7.5 x 10^5, 7.5 x 10^7 cfu SE, 34.5, 65.5 and 100% of the hens were found antibody-positive after 28 days.[27]

The immune response brought about by dosing hens with SE appears to be long-lasting. Humphrey et al.[18] found sustainable peak serum antibody titers up to 70 days post inoculation in 18-week-old specific pathogen free (SPF) White Leghorn hens that had been inoculated with 6 or 8 $\log_{10}$ SE PT 4. Barrow and Lovell[31] found similar results when they inoculated 24-week-old hens with 8.5 $\log_{10}$ SE PT 4. Peak serum antibody titers were maintained for 189 days. It is important to note that the hens used in this study were obtained from a commercial flock and their *Salmonella* history was unknown. Any previous exposure of these birds to *Salmonella* might therefore have influenced the observed results.

In another study, Gast and Beard[25] observed a decline of peak serum antibody titers two weeks post inoculation in 27, 37, and 62 week SPF White Leghorn hens inoculated with 9 $\log_{10}$ SE PT 13a. This decline continued for up to 70 days. These kinetic data are supported by data from Bichler et al.[32] The decline rate was inversely related to the age of the hen. Antibody titers of 62 week old hens fell to 1:68 in 21 days compared with 37- (1:40) and 27- (1:53) week- old hens where similar levels were only seen at 70 days. Conversely, the initial response, antibody serum levels, was not age dependent. Two different *Salmonella* strains (SE PT 31a vs. SE PT 4) were used in the preceding studies and may have accounted for the variability observed between them.

Gast and Beard[28] then investigated the time during which peak levels of antibodies (IgY) could be sustained in the yolk of eggs produced by 27 week old SPF Single-Comb White Leghorn hens inoculated with 9 $\log_{10}$ SE PT 13a (strain: 19299-52-1). They found peak IgY titers were sustained for up to 49 days post inoculation. These data imply serum antibody titers of 27-week-old hens can maintain sufficient levels to endow the yolk of their eggs with peak

antibody levels at least to 49 days, even though serum antibodies levels are declining.[25] Cumulatively these data suggest maternal antibodies hinder SE growth.

Contact exposed hens

Immune responses of hens infected with SE by natural contact with other birds may be less robust than those that follow artificial exposure in the laboratory. Two studies that used the same hen breed and SE strain reported that contact exposed hens had peak antibody titers after three to five weeks in both serum and their egg yolks.[25;28] The immune response was slower than that described above for artificially inoculated birds. Gast and Beard[25] demonstrated that contact birds produced statistically significant ($P = 0.005$) lower levels of serum antibody during the first two weeks than their artificially inoculated counterparts. Both contact and inoculated birds in this study were aged 27 and 37 weeks. Conversely, there was no statistical difference in the levels of IgG observed in contact and artificially infected hens between two and 10 weeks after exposure to *Salmonella*. Gast and Beard[28] further demonstrated that during the second and third week following exposure, 27-week-old contact birds produced lower levels of serum antibody, compared to artificially inoculated hens. Unlike the earlier study,[25] the peak magnitude of the yolk antibody response in the subsequent study[28] was similar to or greater than their artificially inoculated counterparts.

The peak antibody level achieved would depend on the size of the dose, the frequency of exposure, and the route of transmission to the hen. The former two factors are unknowns in a hen contact situation. Differences in the route of transmission, such as by aerosol or orally, will affect the magnitude and incidence of a hen's immune response.[33-35] These factors could thus have contributed to temporal and scale differences observed between the two studies. These two studies together suggest that the dose of SE passed to contact exposed hens would likely be less than that administered to artificially infected hens and that peak antibody levels achieved by artificial inoculation are more variable than those following natural contact are.

Naturally infected hens

To determine the incidence of antibodies in the serum of naturally infected hens, investigators have examined flocks previously associated with SE infection or human outbreaks of salmonellosis. Hoop and Pospischil[36] found 90.0% (10/11) of hens tested from flock 1 (1 year old), 52.5% (11/21) of hens tested from flock 2 (2 years old) and 60.0% (3/5) of hens tested from flock 3 (2 years old) had SE-specific serum antibodies (yolks from these hens' eggs were not assayed), thereby indicating variability in the number of seropositive hens in a flock.

Gast and Beard[28] investigated the incidence of antibodies in the yolks of naturally infected hens' eggs and found between 5 and 22% of yolks tested from five hen houses were SE IgY-positive. Organ culturing and serological testing of birds from the same flock demonstrated that the percentage of yolk positive samples matched or exceeded the percentage of hens identified as SE-positive by organ culturing and serological tests. These data suggest naturally infected hens' eggs have antibodies to SE in the yolks and therefore, that hen seropositivity can predict the presence of IgY in yolk. Nicholas and Andrews[37] examined serum and yolk antibodies of birds from three flocks (Table E15) and found, in contrast, seropositive status was not necessarily predictive of IgY in yolk. The ambiguous correlation between in-flock seropositivity for IgG and

the incidence of IgY in a flock's eggs was also observed by Desmidt et al.,[38] who examined serum and yolk antibodies from birds within the same flock. In that study, when both hens and eggs were randomly picked for sampling, a range of results within flocks was observed. For instance, in flock 1, 100% (20/20) of hens was serum positive and 70% (14/20) of yolks was positive. In flock 2, 20% (2/10) of hens was serum positive and 8.7% (2/23) of yolks was positive. And in flock 3, 46.9% (15/32) of hens was serum-positive and 46.0% (28/50) of yolks was positive.

TABLE E15 IGG DETECTION IN HEN SERUM AND YOLK.[37]

Flock (age, weeks)	House	% serum positive	% yolk positive
A (56)	NA	60 (30/50)	32 (32/100)
B (55)	NA	75 (109/145)	63 (114/180)
D (58)	1	100 (60/60)	35 (21/60)
D	2	90 (54/60)	72 (43/60)
D	3	53 (32/60)	15 (9/60)

Because each of the above studies, i.e. Gast and Beard,[28] Nicholas and Andrews,[37] and Desmidt et al.,[38] used randomly chosen birds and eggs from within the flock, the percent yolk positive statistic likely does not correlate directly to only seropositive birds. That is, columns 3 and 4 of Table E15 are not paired statistics except in the case of Flock D, where all birds tested from house 1 were SE-seropositive. Only 35% of eggs produced by these hens were yolk antibody-positive. These data suggest that naturally seropositive hens deposit maternal antibodies into the yolk, although the seropositivity of a hen is not a predictor of yolks with antibodies.

SE growth in the presence of yolk antibodies

Perhaps the most direct evidence for suppression of SE growth by an immune response comes from four studies examining growth of SE in the presence of yolk antibodies (IgY) specific for SE. Two studies investigated SE growth directly in IgY-containing yolk and two investigated SE growth with IgY in neutral media. Bradshaw et al.[9] inoculated yolks from naturally seropositive SE hens with 10 cfu SE at 37°C. Growth in this environment was slower compared to when SE were inoculated into seronegative hen yolks, suggesting the presence of SE-specific IgG could attenuate SE growth within yolk. Conversely, the study of Takase et al.,[39] in which diluted yolks from SE immunized hens were inoculated with 10 cfu SE and incubated at 37°C, did not show a significant difference between the growth of SE in yolks of seropositive and seronegative hens. Neither study assayed for yolk SE-specific antibodies; therefore, the IgY levels are unknown. In addition, neither study described the methods used for determining serum antibody titers, making any direct comparison difficult.

Two studies were identified that investigated SE growth in the presence of IgY. Lee et al.[40] purified IgY from SE-immunized hens. The IgY (5.4 mg) thus obtained was incubated with 7 $\log_{10}$ cfu SE resulting in attenuated SE growth post 4 hours compared to control IgY (IgG not specific for SE). Conversely, Sugita-Konishi et al.[41] purified SE-specific IgY from SE-immunized hens. Incubation of 4 $\log_{10}$ cfu SE with 1 to 10 mg IgY did not affect growth of SE over 24 hrs. Lack of reported detail regarding methodology made it difficult to compare directly the observed results.

Nevertheless, despite these conflicting reports, there appears to be some effect of yolk IgG on growth of SE in eggs. Admittedly, it is difficult to estimate the effect of an antibody binding to a bacterium under conditions within an egg: typically, antibodies work in conjunction with complement factors and macrophages, elements that are not present in the egg; therefore, the effect of antibody-mediated cytotoxicity will be greatly diminished *in vivo*. Yet this is not expected to negate antibody effect on SE growth within yolk, as antibody binding could disrupt a bacterium's ability to obtain nutrients and grow.

Because data from these studies were neither consistent nor complete, they were not used explicitly for modeling; though data from Bradshaw et al.[9] indicating SE growth was suppressed in seropositive hens were included.

Attachment E1: Equations for computing growth of SE in Egg yolk and vitelline membrane

Deterministic growth parameters alone may not adequately describe growth from small initial densities of bacteria.[42] For SE contamination of shell eggs, initial densities are believed to be less than 10 SE per egg.[3] Therefore, the random or stochastic nature of bacterial growth is accounted for in this attachment under two topics: (i) development of Equations EA1 to EA10 and (ii) application of Equations EA11 and EA12 in growth modeling.

Development of equations for stochastic growth

The derivation for the exact equations for stochastic growth described below was recently published.[43] It is assumed that a given bacterial cell goes through a lag phase before an exponential phase of growth and that the duration of the lag phase is distributed with cumulative distribution function, $H(t)$, where t is time. Very little information is available concerning the distribution H, and, as a default, it is assumed that H is an exponential distribution. Chea et al.,[44] in a study on *Clostridium botulinum*, stated that, for generation times, the distribution appeared to be a Weibull distribution, but that an exponential distribution did provide a reasonable fit. Of course, this may not reflect the situation for SE in eggs.

The equations that describe growth, starting with one cell, are derived from the general theory of stochastic processes[45] and are developed[46] as follows:

Let t_0 be the time that the original (*O*-cell) becomes a cell in exponential phase (*D*-cell) with an assumed exponential growth rate of $\mu(t)$, and let $\nu(t) = \int_0^t \mu(\tau)d\tau$. In the following, the exponential growth rates are assumed to be known functions of time. Then the distribution of the increase in the number of *D*-cells, $I(t)$, associated with the one *D*-cell at time $t > t_0$, is the geometric distribution [45]with generating function

$$g_I(s,t,t_0) = \frac{e^{-\nu(t,t_0)}}{1-\left[1-e^{-\nu(t,t_0)}\right]s}. \qquad \text{(EA1)}$$

where $\nu(t, t_0) = \nu(t) - \nu(t_0)$. Thus, conditional on the time, t_0, the generating function, $F(s,t|t_0)$, for the number of cells, from a given original cell, is:

$$F(s,t \mid t_0) = \iota(t < t_0)s + \iota(t \geq t_0)[s g_I(s,t,t_0)] \qquad \text{(EA2)}$$

where $\iota()$ is equal to 1 if the argument is true; otherwise it is equal to 0. Let $H(t) = Prob(t_0 < t) = 1 - e^{-\gamma(t)}$ be the cdf of the transition time, where $\lambda(t)$ is the instantaneous probability (or hazard function) of an *O*-cell becoming a *D*-cell, and $\gamma(t) = \int_0^t \lambda(\tau) d\tau$. The unconditional generating function of the number of cells $F(s, t)$ is:

$$F(s,t) = s\, e^{-\gamma(t)} + \int_0^t s\, g(s,t,\tau)\, dH(\tau) \qquad \text{(EA3)}$$

where $dH(\tau) = \lambda(\tau)e^{-\gamma(\tau)}\, d\tau$.

The expected value and the variance of the relative growth, $r(t)$, can be obtained, using the properties of generating functions, as,

$$\begin{aligned} E(r(t)) &= e^{-\gamma(t)} + \int_0^t e^{\nu(t,\tau)} dH(\tau) \\ var(r(t)) &= 2\int_0^t e^{2\nu(t,\tau)} - e^{\nu(t,\tau)}\, dH(\tau) + E(r(t))(1 - E(r(t))) \end{aligned} \qquad \text{(EA4)}$$

The probability of j organisms at time is derived from Equation EA3:

$$\begin{aligned} p_1(t) &= e^{-\gamma(t)} + \int_0^t e^{-\nu(t,\tau)} dH(\tau) \\ p_j(t) &= \int_0^t e^{-\nu(t,\tau)}(1 - e^{-\nu(t,\tau)})^{j-1} dH(\tau), \quad j > 1 \end{aligned} \qquad \text{(EA5)}$$

When the number of cells at $t = 0$, S_0, is greater than 1, the generating function of the number of organisms is $(F(s, t))^{S0}$, so that computing the probabilities would become difficult with even a moderate number N_0.

In this application, it is assumed that the product of $\lambda(t)$ and $\mu(t)$ is constant. This is based on observations by Ross[6] that the ratios of the mathematical lag times (defined as the intersection of the horizontal line, $y = s_0 = \log_{10}(S_0)$, and the tangent line of the growth curve at the point of maximum exponential growth rate, as t approaches infinity) to generation times (= $\ln(2)/\mu$) are nearly the same for different fixed conditions of growth for many organisms. Thus, assuming a constant temperature, λ and μ are constants, and the mathematical lag time, *lagGrowth*, is

$$lagGrowth = \frac{\ln(1+\mu/\lambda)}{\mu}. \quad \text{(EA6)}$$

Thus the ratio of the lag growth to the generation time is:

$$Rat = \frac{\ln(1+\mu/\lambda)}{\ln(2)}. \quad \text{(EA7)}$$

For growth of SE in eggs, information concerning lag phase duration times is not available. The maximum slope of the growth curve used by Ross[6] and the exponential growth rates described in the above equations may not be the same because many of the growth curves that were used included Gompertz equations. However, it is expected that the differences would not be great and are close enough to permit the above relations to hold in approximation. Thus, from Equation EA7, assuming a value of *Rat*, for a given value of $\mu(t)$, the corresponding value of $\lambda(t)$ can be computed.

The above set of equations holds when cells are not beyond the exponential phase of growth and when the assumption that cellular events are independent is valid. However, for large numbers of cells, near the maximum level where the rate of growth decreases, the independent assumption is clearly not valid. For a large number of cells, the variability of growth would be small, and thus could be ignored without much loss in accuracy. If it is assumed that a large proportion of cells are out of lag phase at time t_0, then the growth of the number of cells, $S(s)$, for $s > t_0$, is assumed to be described by a logistic growth curve, with differential equation,

$$\frac{dS(s)}{ds} = \mu(s)S(s)\left[1-\frac{S(s)}{M}\right] \quad \text{(EA8)}$$

where M is the maximum level. The solution of this differential equation is

$$S(t) = \frac{S(t_0)e^{\nu(t)}}{1+\frac{S(t_0)}{M}(e^{\nu(t)}-1)} \quad \text{A9}$$

For application, note that equations for the expected value and the probability of no growth for a given cell are the same except for the minus sign in the exponent of e in the integral. Now, if there are S_0 cells, the variance of $r(t)$ is computed as:

$$S_0 \operatorname{var}(r(t)) = 2\int_0^t \left[e^{2\nu(t,s)} - e^{\nu(t,s)} \right] e^{-\gamma(s)} ds + E(r(t))[1 - E(r(t))]$$

(EA10)

Application of stochastic equations to the risk assessment growth model

The above set of equations supports a three-stage approach for determining the growth of SE in contaminated eggs, which accounts for inherent variability of that growth for small numbers of initial SE cells. The first stage distinguishes between when there is growth and when there is not. The second stage approximates the amount of growth using a lognormal distribution. The third stage accounts for growth when bacterial levels approach the stationary phase.

For the first stage, the probability of growth at time t is q = 1- $p_1(t)^{S_0}$. Thus, in the simulations for the risk assessment, 1-q percent of the time there would be no growth and q percent of the time there would be. For the second stage, when growth occurs in stage 1, then the conditional expected value of $r(t)$ is computed, given that $r(t)$ is greater than 1, $E_g(t)$. This is derived as:

$$E_g(t) = \frac{E(t) - (1-q)}{q}$$

(EA11)

where $E(t)$ is the unconditional expected value $E(r(t))$ given in Equation EA4 and q is the probability that $r(t)$ >1. Similarly, the variance of $r(t)$) given that $r(t)$ >1, $V_g(t)$, can be computed from

$$V_g(t) = \frac{V(t) - (1-q)q^{-1}(E(t)-1)^2}{q}$$

(EA12)

where $V(t)$ is the unconditional variance, $var(r(t))$ computed from Equation EA9. Equation EA12 was derived using the relationship: $var(x) = E(var(x|y) + var(E(x|y))$, where x and y are random variables. The number of cells at t is approximated from a lognormal distribution with (conditional) mean and variance given in Equations E11 and E12. The lognormal distribution is expected to provide a reasonable approximation for moderate amounts of increase.[47] Further, an example growth scenario was developed for comparing the distribution of the number of cells, conditional on there being growth, using the exact equations developed above (Equation EA5) and the lognormal approximation. In the example, a common temperature is assumed, so that λ (= 0.5) and μ (= 6.9) are constant for some unspecified unit of time. Figure E-A1 is an example,

assuming $t = 1$, where the exact solution was compared with the lognormal approximation , with parameters determined by equating the first and second moments, using Equations EA11 and EA12.

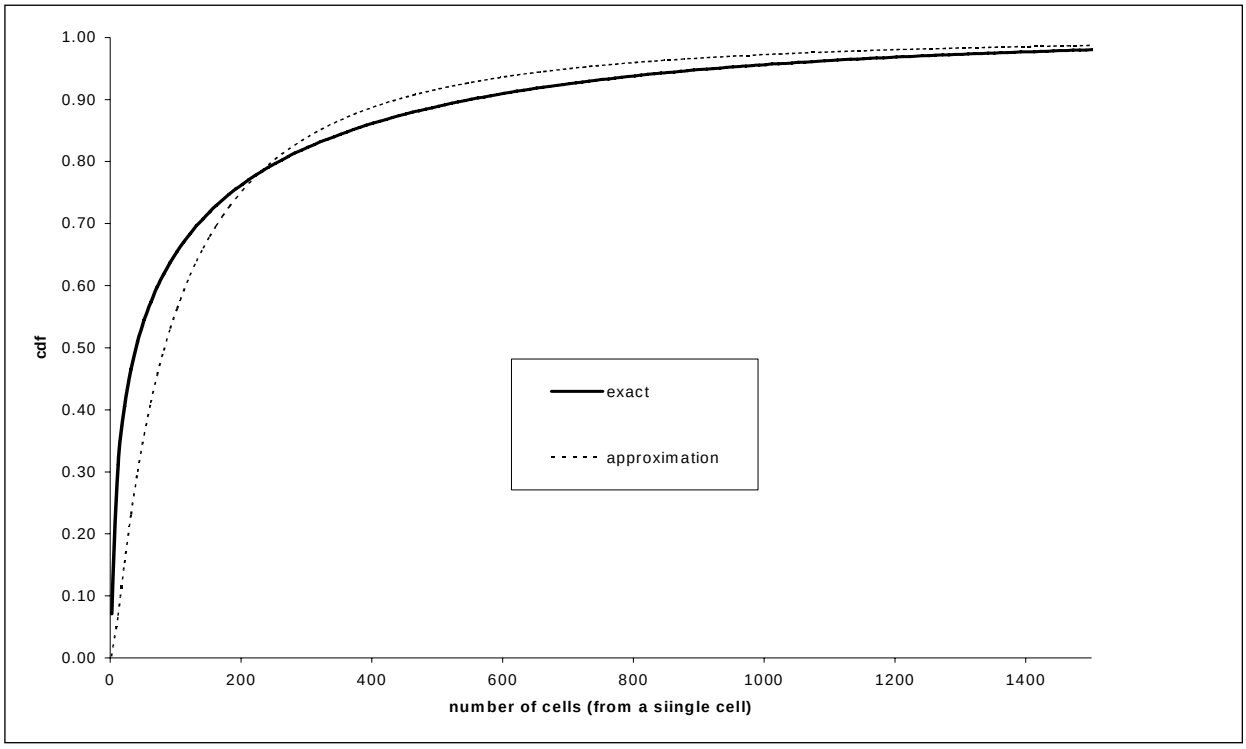


FIGURE E-A1 COMPARISON OF EXACT SOLUTION AND APPROXIMATION USING LOGNORMAL DISTRIBUTION OF THE NUMBER OF CELLS GROWING FROM A SINGLE CELL.

However, if the generated number is near M, then an adjustment for the logistic growth is needed. This can be done by considering a time, $t_0 < t$, and computing the expected value, $E_g(t_0)$ and the variance $V_g(t_0)$ from Equations EA11 and EA12, assuring that $E_g(t_0)$ is large but not too close to M, and then generating a random variable, representing the number of cells at time t_0. Then Equation EA9 is used.

Modeling growth of *Salmonella* spp. in Egg Products

Unlike the available data for growth of SE in shell eggs, the data for modeling *Salmonella* spp. growth in egg products as functions of times and temperatures were too sparse to permit explicit modeling. Baron et. al.[48] reported rapid growth of SE in reconstituted dry egg white after one condition of time-temperature (24 hours at 30ºC). SE in reconstituted dried albumen increased 5 $\log_{10}$ cfu/mL relative to an increase of 1 $\log_{10}$ cfu/mL for untreated liquid albumen in 24 hours at 30ºC. The authors hypothesized that the rapid growth reflects loss of activity of the normally

inhibitory substances in intact albumen by protein denaturation resulting from the drying process. Denaturation of ovotransferrin was associated with the rapid growth in dried albumen.

The possibility cannot be dismissed for rapid SE growth in egg products before and after pasteurization. It is also possible that SE surviving pasteurization are injured and require a longer lag period before growth could commence. Because no quantitative data exist to describe these processes explicitly, the rate of growth in egg products was modeled as described for the rate of growth in yolk. The lag was considered a state of knowledge factor 0 to 10X that of the lags determined for growth rates of SE in egg yolks.

TABLE E-A1 DATA REGARDING SE GROWTH IN ARTIFICIALLY INOCULATED EGG ALBUMEN.[A]

Reference	Experiment performed in:	Age of egg (days)[b]	Incubation time (days)[c]	Temp (°C)	Inoculum (SE)	Location of SE within egg[d]	Enumerated from:	Results
Baker 1990	Isolated albumen	1	0-19	37	1.2 log_{10} cfu/sample 3.2 log_{10} cfu/sample	NA	Albumen	SE persisted up to 7 d in samples of 1.2 log_{10} cfu. In samples of 3.2 log_{10} cfu, SE persisted for 19 d followed by decline in samples post-5 d.
Bradshaw et al.[9]	Isolated albumen	< 7, held at 4°C	2	37	3.6 log_{10} cfu/g[e]	NA	Albumen	No growth, observed decline.
Lock and Board[49]	Isolated albumen	< 2-4 1	1-42	20	3 log_{10} cfu/sample	NA	Albumen	15% (2/13) persisted 1 d: 2.3 fold growth (0.36 1og10±0.02).
Humphrey and Whitehead[2]	Isolated albumen	42	5	20	2.7 log_{10} cfu/sample	*Eac*[f]	Albumen	42 d: 4.9 fold growth (0.68 1og10±0.09)
		42	5	20	2.7 log_{10} cfu/sample	*Eaf*[f]	Albumen	42 d: 1.9 fold growth (0.28 1og10±0.01)
Gast and Holt[16]	Isolated albumen	1	3	25	1.2 log_{10} cfu/ sample 2.2 log_{10} cfu/ sample	NA	Albumen	no growth for both inoculum sizes.
Gast and Holt[12]	Isolated albumen	1	3	25	5 log_{10} cfu/ml[e]	NA	Albumen	no growth in 0/120 samples, observed decline.
Clay and Board[50]	Whole eggs, intact shell (IS)[g]	< 4	8	25	1 log_{10} cfu/egg	*Eaf*[h]	Albumen	no growth (> 1 log_{10} cfu/ml) in 0/12 of samples.

Humphrey et al. 1991	Whole egg, IS	< 1	5	20	2 $\log_{10}$ cfu/egg	NA	Albumen	no growth in 0/20 samples
Humphrey[4]	Whole eggs, IS	0.17	1 -34	20	2.7 $\log_{10}$ cfu/egg	*Eac*	Albumen	No consistent growth b/t 1-19 days.
Humphrey, 1998	Whole eggs, IS	0.17	1.5 - 35.5	20	0-0.48 $\log_{10}$ cfu/egg	NA	Albumen or albumen and yolk mixed, unknown	No consistent growth b/t 1.5-26.5 days.
Hara-Kudo et al.[51]	Whole eggs[i]	27	3	20	0.9-1.3 $\log_{10}$ cfu/egg	*Eac*	Albumen and yolk mixed	No growth (> 1 $\log_{10}$ cfu/ml).
Schoeni et al.[14]	Whole egg IS	3	1	10, 4	3.5, 5.5 $\log_{10}$ cfu/egg	*Eaf*	Albumen and yolk separately	Decline in albumen at 10ºC; Decline in both albumen and yolk at 4ºC 46% (6/13) w/ generation times of 2-12 d. 38% (/13) w/ generation times of 13-19 d.
Lock and Board[49]	Isolated albumen	< 2-4	1-42	20	3 $\log_{10}$ cfu/sample	NA	Albumen	
Cogan et al.[3,7]	Isolated albumen	< 0.25	8	20	0.3, 1.4, 2.4, 3.4 $\log_{10}$ cfu/ 25 ml sample (-1, 0, 1, 2 $\log_{10}$ cfu/mL)	NA	Albumen	Growth (> 4 $\log_{10}$ cfu/ml) occurred ca.2.5%, 3.5%, 32.5% and 60.0% with inoculums 0.3, 1.4, 2.4, 3.4 $\log_{10}$ cfu/sample, respectively. See Figure E3.

								Eleven SE and 6 non-SE strains tested; "on the average", both SE and non-SE strains grew well in fresh albumen; in fresh albumen by 23 days PI, non-SE strains grew more than SE strains; some strains grew better in isolated albumen.
Duboccage et al.[52]	Isolated albumen	"Fresh" egg but unspecified age OR 2-week old or 3-week old isolated albumen	6, 13, or 23 days	20	2 log_{10} cfu/mL	NA	Albumen	from fresh eggs, others in albumen from eggs stored for 2-3 weeks.
Baron et al.[48]	Isolated albumen or reconstituted commercial egg white powder	3 to 10	1	30	3 log_{10} cfu/mL	NA	Albumen	ca.1 log10 increase for isolated albumen; ca.5 log10 increase for reconstituted albumen (versus ca. 6 log10 increase for tryptone soy broth)
Kim et al.[53]	Whole eggs, intact shell (IS)[f]	1	10	21	2.7 log_{10} cfu/egg 3.7 log_{10} cfu/egg	*Eaf* or *Eac*, unknown *Eac*[g]	Albumen and yolk mixed	2.7 log_{10} cfu/egg: growth of 3.6 log_{10} cfu /ml. 3.7 log_{10} cfu/egg: growth of 5.0 log_{10} cfu /ml. Eac: growth (> 1 log_{10} cfu /ml) in 16.7% of samples (2/12).
Clay and Board[50]	Whole eggs, IS	< 4	8	25	1 log_{10} cfu/egg	*Eaf*[g]		E*af*: growth (> 1 log_{10} cfu /ml) in 0% of samples (0/12).

							Albumen	
						Eac[g]		E*ac*: growth (> 1 log_{10} cfu /ml) in 31% of samples (10/32).
						Eaf[g]		E*af*: growth (> 1 log_{10} cfu /ml) in 15.6% of samples (5/32).
	Whole eggs, IS	< 4	8	25	3 log_{10} cfu/egg		Albumen	
						Eac[g]		E*ac*: growth (> 1 log_{10} cfu /ml) in 50% of samples (6/12).
						Eaf[g]		E*af*: growth (> 1 log_{10} cfu /ml) in 25% of samples (3/12).
	Whole eggs, IS	< 4	8	25	6 log_{10} cfu/egg		Albumen	
						Eac	Albumen	*Eac*: growth of 9.5 1og10/egg[h].
						Eaf		E*af*: growth of 0.6 1og10/egg.
	Whole egg, IS	14	5	20	2 log_{10} cfu /egg			
Humphrey et al.[54]						*Eaf* or *Eac*, unknown		>3 log_{10}: growth (unknown) in 3/20 samples.
	Whole egg, IS	< 1	5	20	> 3 log_{10} cfu /egg		Albumen	
Humphrey[8]								
	Whole eggs[i]	< 0.08	4	20	2.7 log_{10} cfu /egg	*Eac*	Albumen	See Figure E1.
	Whole eggs[i]	< 0.08	0.41-1	20	2.7 log_{10} cfu /egg	*Eac*	Albumen	Growth of 1 log_{10}.
		1	5	20	2.7 log_{10} cfu /egg	*Eac*	Albumen	1 d: mean growth: 1.1 log_{10} cfu/egg range: (0.6-2.4 log_{10} cfu/egg).
		21						21 d: mean growth: 1.5 log_{10} cfu/egg range: (0-4.9 log_{10} cfu/egg).
Humphrey and	Whole eggs[i]							

Whitehead[2]								
Humphrey[4]	Whole eggs, IS	0.17	1	20	2.7 log_{10} cfu /egg 3.5 log_{10} cfu /egg	*Eac*	Albumen	Growth of 1 log_{10}. 3.5 log_{10}: Growth of 6.5 log_{10} cfu/egg[h].
Schoeni et al.[14]	Whole eggs, IS	3	1	25	5.5 log_{10} cfu /egg	*Eaf*	Albumen	5.5 log_{10}: Growth of 4.5 log_{10} cfu /egg[h].
Gast and Holt[16]	Whole eggs, IS	1	3	25	2.2 log_{10} cfu /egg	*Eaf*	Albumen and yolk mixed	Growth of ca. 0.8 log_{10} cfu/ml.
		6						6 d: growth (ca. 1 log_{10} cfu/egg) in 10% of samples (2/20). 20 d: growth (ca. 1-1.5 log_{10} cfu/egg) in 62% of samples (13/26).
Hara-Kudo et al.[51]	Whole eggs[i]	20	3	20	0.9-1.3 log_{10} cfu/egg	*Eac*	Albumen and yolk mixed	
Cogan et al.[5,3,7]	Whole eggs	< 0.25 hrs	8	20, 30	0.3, 1.4, 2.4, 3.4 log_{10} cfu/egg	*Eac*	Albumen	See Figure E2 A,B

a. Growth defined as positive difference in final SE density minus starting inoculum.
b. Age of egg at inoculation.
c. Incubation time of inoculated egg before analysis.
d. *Eaf*: deposition of SE within outer albumen, *Eac*: deposition of SE within albumen, close to yolk.
e. albumen typically 35-40ml and ca. 40g.
f. The location from which the albumen sample was removed from the egg.
g. Whole egg artificially inoculated through shell.
h. *Eaf*: SE inoculated into egg air cell, egg placed broad end down; yolk floats away from air cell.
i. Whole egg contents broken into container and inoculated.
j. Growth defined as positive difference in final SE density minus starting inoculum.
k. Age of egg at inoculation.
l. Incubation time of inoculated egg before analysis.
m. *Eaf*: inoculation of SE within outer albumen, *Eac*: inoculation of SE within albumen, close to yolk.
n. whole egg: ca. 55 ml; albumen: 35-40 ml.
o. Whole egg inoculated through shell.

p. *Eaf*: SE inoculated into egg air cell, egg placed broad end down; yolk floats away from air cell. E*ac*: SE inoculated into egg air cell, egg placed small end down; yolk floats toward air cell.
q. Evidence of yolk membrane breakdown likely due to age of sample or high inoculum level.
r. Whole egg contents broken into container and inoculated.
s. The definitive study used for modeling the probability and extent of growth in albumen was that of Cogan[7]

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