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Egg age and the growth of *Salmonella enteritidis* PT4 in egg contents

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SUMMARY

The growth of *Salmonella enteritidis* PT4 in albumen around an intact yolk was governed by the age of the egg on inoculation. In the majority of eggs, held at 20 °C, the bacterium was unable to grow rapidly until eggs had been stored for approximately 3 weeks. The multiplication of *S. enteritidis* in stored eggs appeared to be associated with alterations to the yolk membrane which allowed the bacterium to either invade the yolk or obtain nutrients from it. The rate at which egg contents change to permit the growth of *S. enteritidis* would appear to be temperature related and took place more rapidly when eggs were stored under conditions where temperatures fluctuated and, on occasions, reached 30 °C.

INTRODUCTION

A large number of studies have been carried out on the impact of storage on the microbiology of egg contents. Until recently, research concentrated upon spoilage organisms [1] although some work was undertaken on the growth of salmonellas [2–4]. The current association between human infections with *Salmonella enteritidis* and contamination of eggs [5] has meant that attention has been focused on conditions which influence the number of cells of this bacterium in egg contents.

Current scientific evidence would suggest that the number of *S. enteritidis* in the great majority of contaminated contents of clean, intact, fresh eggs from either naturally [6–9] or artificially infected [10, 11] laying hens is low. This is in line with previous observations on eggs laid by hens infected with *S. pullorum* [12]. Recent work [8] has demonstrated a highly significant association between the age of an egg and the number of *S. enteritidis* present in egg contents. Thus, with naturally contaminated eggs stored at 20 °C, all those examined within 3 weeks of lay contained less than 20 cells, whereas over 50% of positive eggs that were 3 weeks old or older contained more than 100 cells of *S. enteritidis*. In separate studies, only minor changes in levels of contamination were observed in eggs from hens infected artificially with *S. enteritidis* and stored at room temperature for 7 days [11].

Examination of eggs from naturally [8] and artificially infected [11] hens has revealed that the principal sites of contamination of egg contents with *S. enteritidis* are either the outside of the membrane surrounding the yolk (vitelline membrane) or in the albumen and not yolk contents. This direct evidence is

supported by the observation, referred to earlier [8], that there is a delay, in the majority of eggs, before *S. enteritidis* is able to grow well. When inoculated into the yolk of an 'intact' egg, the organism grows from a low inoculum (< 5 cells per egg), in eggs of any age, whether they come from infected or uninfected hens [8, 13]. These results are essentially the same as those from earlier studies with other salmonellas [14] and could be taken to suggest that, if yolk contents were the principal site of contamination, the majority of salmonella-positive eggs would be heavily contaminated irrespective of egg age. This is not the case [6–11].

There are a number of possible explanations for the delay seen in the growth of *S. enteritidis* in contaminated eggs [8]. An important controlling factor on the growth of salmonellas in albumen is iron limitation [15]. It is possible that during storage, changes in egg contents increase the availability of iron possibly as a result of alterations to the integrity of the vitelline membrane [16–17] allowing either access to or the release of iron in the yolk. Alternatively it may be that the inherently inhibitory properties of egg albumen lessen with egg age.

Experiments were performed to assess the potential importance of possible storage-related changes in allowing the rapid growth of *S. enteritidis* in egg contents. The results of these studies, which include some data on the effect of storage temperature, are presented in this paper.

MATERIALS AND METHODS

Eggs

Eggs were obtained, within 1–2 h of lay, from a small local battery farm with 5000 ISA-brown laying hens. Repeated testing by this laboratory and MAFF had demonstrated that hens and eggs from this source were free from *S. enteritidis*. Staff collecting the eggs were asked to take only those that were free from faecal contamination, still warm and apparently intact.

Bacterial strain and culture

A strain of *S. enteritidis* phage type (PT) 4 isolated from egg contents was used in all the experiments described in this paper. The organism was grown in static culture in Lemco broth (Oxoid) at 37 °C for 18–24 h and dilutions made in Ringer's solution before inoculation into egg contents.

Experimental protocols

On arrival at the laboratory, eggs were examined individually, and all those that were either cracked and/or contaminated with faeces were discarded. The remaining eggs were used in the experiments described below.

The impact of storage at 20 °C on the ability of albumen in whole egg contents to support the growth of S. enteritidis

A total of nine experiments was carried out. On each occasion approximately 300 fresh eggs were placed in an incubator set at 20 ± 0·5 °C. At intervals of 2–3 days, between 20 and 60 eggs were removed and examined for the ability of albumen in the area around the intact yolk to support growth of *S. enteritidis*. For this purpose, egg contents were broken out, using aseptic techniques, into sterile,

60 ml plastic screw-capped containers (Metlab Ltd). Albumen was inoculated, using an automatic pipette, with 500 cells of *S. enteritidis* PT4 in 0·1 ml Ringer's solution. The inoculated eggs were held at 20 ± 0·5 °C for 5 days and the numbers of salmonellas in each egg were then estimated by plating egg homogenates on XLD agar (Oxoid), incubated at 37 ± 0·5 °C for 18–24 h. When little or no growth was expected, each of 10 XLD plates was inoculated with 0·6 ml (15 drops from a sterile '25' dropper pipette). When growth was either expected or apparent in the contaminated eggs, only one plate per egg was used. Assuming that the majority of eggs held 50 ml of contents, minimum levels of detection using these two techniques were 8 and 83 cells of *S. enteritidis* per egg respectively. All homogenates were held at +4 °C while the plates were incubated. If the numbers of salmonellas on any of the XLD plates were too high to permit accurate estimation, the stored homogenates were diluted in Ringer's solution to 10^{-7} and plated on XLD using standard techniques. Growth of salmonellas in the egg contents was expressed as a ratio of the number of cells present after 5 days at 20 °C (T5) over the inoculum (T0). The T5/T0 figures for the 20–60 individual eggs were converted to logarithms and used to calculate the geometric means for each sampling time.

Storage temperature fluctuations and the growth of S. enteritidis *in albumen of whole egg contents*

A small survey of local shops revealed that, in some cases, eggs were displayed for sale in shop windows and were exposed to direct sunlight. To examine the effect of this, 200 eggs were held on a laboratory windowsill where they were in sunlight for up to 4 h per day and temperatures fluctuated between 18 and 30 °C. At 2–3 day intervals, eggs were examined for the ability of albumen around the intact yolk to support the growth of *S. enteritidis* from an initial inoculum of *c.* 500 cells. The techniques used were the same as those described earlier. Two replicate experiments were carried out.

Growth in separated egg albumen

Three separate experiments were performed. The contents of 20 intact, fresh eggs were broken into honey jars and stored for either 1 day or 6 weeks at 20 ± 0·5 °C. After storage, a piece of sterile, plastic pipe was placed around each yolk so that a band of albumen with an approximate width of 1 cm was enclosed within the piping. This albumen, usually 7–10 ml, and an equivalent volume from outside the ring of plastic, was removed with sterile wide-bore glass 10 ml pipettes and placed in separate screw-capped 25 ml bottles. These were inoculated with sufficient cells of *S. enteritidis* to achieve contamination levels of 10 cells per ml and held at 20 ± 0·5 °C for 5 days. The number of salmonellas was estimated, as before, with the exception that each sample of albumen was homogenized with 50 ml BPW before plating.

Experiments to investigate the relative importance of changes to either membrane permeability or albumen

In three separate experiments, the contents of 60 eggs were broken into honey jars and stored at 20 ± 0·5 °C for either 1 day or 6 weeks. Twenty eggs were subjected to no further treatment before inoculation. With 20 others, that

were 6 weeks old, all the albumen or that from an area around the yolk was removed, using the technique described above, and replaced with equivalent albumen from 20 fresh eggs and vice versa. All eggs were inoculated, into the albumen next to the yolk, with *c.* 500 cells of *S. enteritidis* and held at 20±0·5 °C for 5 days. Relative growth rates were calculated as described earlier.

The influence of egg storage at 20 °C on the ability of S. enteritidis *to invade the contents of intact yolks*

Twenty fresh eggs were placed at 20±0·5 °C for either 1, 21 or 42 days. Eggs were inoculated into the albumen next to the yolk with 500 cells of *S. enteritidis* using the technique described earlier. The inoculated eggs were held at 20 °C for 5 days. The albumen from each egg was removed, using aseptic techniques, and placed in a separate sterile 100 ml screw-capped plastic container. Each intact yolk was washed with 40 ml of sterile buffered peptone water (BPW). This was removed and added to the albumen of the appropriate egg. The outer surface of the intact yolk was disinfected by immersion in 70% industrial methylated spirits (IMS) for 10 mins. The IMS was removed and yolk contents were homogenized with 20 ml BPW. The number of salmonellas in each sample of either albumen or yolk was estimated using the plating techniques described above. These experiments were carried out on three separate occasions.

Statistical analysis

The significance in the differences in growth parameters of *S. enteritidis* in stored or fresh eggs was measured using either paired or unpaired *t* tests as appropriate.

RESULTS

Storage at 20 °C and the growth of Salmonella enteritidis *in albumen of whole egg contents*

When *S. enteritidis* was inoculated into albumen around the yolks of fresh eggs (< 2 h old) there was an approximate tenfold increase in the number of salmonellas during the first 10–24 h after inoculation (Fig. 1). Growth rate was independent of the size of the inoculum (data not shown). The pH of the albumen was approximately pH 7·3 on inoculation and rose to pH 9·2–9·4 during the next 24 h.

Once this initial multiplication had ceased there was a delay, in the great majority of eggs (> 90%), before *S. enteritidis* was able to grow rapidly and relative growth rates (T5/T0) increased only slowly until eggs had been stored for 3–4 weeks before inoculation (Table 1, Fig. 1). After this time, *S. enteritidis* was able to grow rapidly in an increasing proportion of the sample population (Table 1). There was good agreement between replicate experiments. The overall means relative growth rates (±S.E.) are shown in Table 1. Data from an individual experiment are presented in Fig. 1.

In a minority of eggs, *S. enteritidis* grew rapidly in albumen, irrespective of egg age. Alternatively, the inoculum either did not grow, but remained viable, or died soon after inoculation.

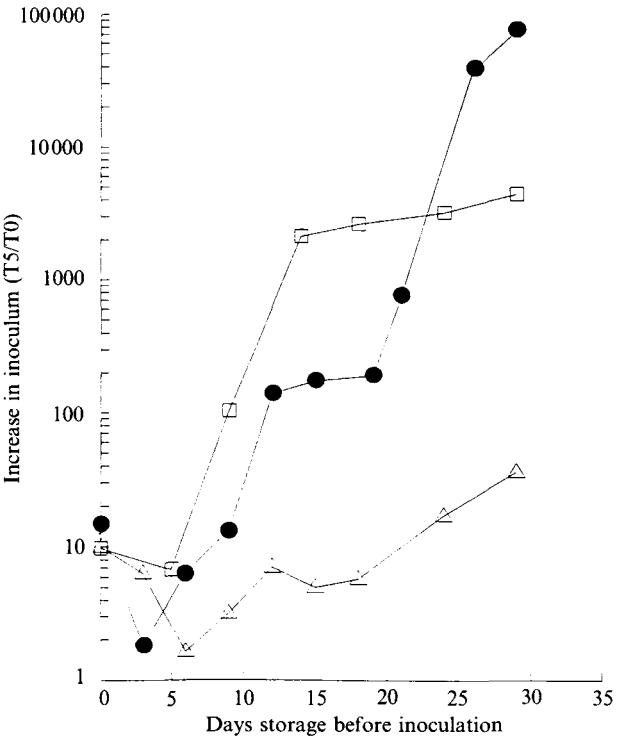

Fig. 1. The influence of storage conditions on the growth of *Salmonella enteritidis* PT4 in the area of albumen next to the yolk. At intervals during the storage period 20 eggs were broken into sterile containers and the albumen inoculated with *c.* 500 cells of *S. enteritidis*. Inoculated eggs were held at 20 °C for 5 days before the numbers of salmonellas were estimated. Relative growth rates were calculated by dividing the numbers of salmonellas after incubation by the inoculum T5/T0. Δ, eggs stored at 20 °C before inoculation; ●, eggs stored under conditions where temperatures fluctuated between 18 and 30 °C (July) before inoculation; □, eggs held under similar conditions in September.

Table 1. *Storage of intact eggs at 20 °C and ability of* Salmonella enteritidis *to grow in egg contents*

Days storage prior to inoculation with *S. enteritidis*	Increase (±S.E.) in the number of salmonellas per egg*	% eggs where increase in inoculum exceeded $\log_{10}$ 3·0
0	9·1±2·75	2·7
7	4·7±2·3	5·0
14	7·5±2·7	4·0
21	15·0±3·4	10·0
28	40·3±2·4†	35·0
42	$> 10^6$†	87·0

* Inoculum was *c.* 500 cells per egg. Eggs were held at 20±0·5 °C for 5 days after inoculation.

† Growth rates in eggs that were either 28 or 42 days old were significantly more rapid ($P < 0{\cdot}01$) than in fresher eggs.

Data are mean values from up to nine separate experiments. In each experiment 20–60 eggs were examined at each sampling point.

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Storage temperature fluctuations and their impact on the ability of albumen of whole egg contents to support the growth of Salmonella enteritidis

When eggs were stored under conditions where temperatures fluctuated between 18 and 30 °C, the storage-related changes which facilitated the growth of *S. enteritidis* took place more quickly (Fig. 1) and after only 10–14 days storage populations exceeding $\log_{10}$ 6·0 cells were achieved in the majority of inoculated eggs. There was, however, greater egg-to-egg variation in these experiments than when eggs were held at a constant 20 °C. For example, in one experiment, carried out in July 1992, the mean c.f.u./egg of *S. enteritidis* in eggs inoculated after 12 days storage was $\log_{10}$ 4·21 $\pm$ 0·66. Counts per egg ranged from $\log_{10}$ 1·92 to $\log_{10}$ 8·92. Substantial growth ($> \log_{10}$ 6·0 per egg) of the bacterium was recorded in 14 of the 20 eggs tested. After 21 days storage, the mean c.f.u./egg had increased to $\log_{10}$ 7·45 $\pm$ 0·84. Counts per egg ranged from $\log_{10}$ 2·1 to 10·7. High bacterial counts ($> \log_{10}$ 6·0 per egg) were observed in 18 of the 20 eggs tested.

Growth of Salmonella enteritidis in albumen

In general, *S. enteritidis* did not grow well in samples of albumen whether they had been removed from near to or away from the yolk of either fresh eggs or ones that had been stored at 20 °C for 6 weeks (Table 2). Even in albumen removed from around the yolk of stored eggs, the numbers of *S. enteritidis* only increased four to fivefold during the 5-day incubation period. This is in marked contrast to the very large population achieved in this albumen when it remained next to the yolk (Tables 1 and 2).

The relative importance of changes in either the vitelline membrane or the albumen in eggs held at 20 °C

In the experiments where albumen was exchanged between fresh and stored eggs, the age of the yolk was found to be the principal factor controlling the growth of *S. enteritidis*. Thus the bacterium grew well in albumen next to yolks from eggs that had been stored for 6 weeks before inoculation irrespective of whether the yolk was surrounded by albumen from fresh or 'stored' eggs (Table 2). Little or no growth occurred in albumen next to yolks from eggs that were 1 day old. As above, growth rates were unaffected by the age of the albumen and *S. enteritidis* was unable to grow, to any significant extent, when albumen from 'fresh' eggs was either wholly or partly replaced by that from stored eggs (Table 2).

The impact of egg storage at 20 °C on the ability of Salmonella enteritidis *to invade the contents of intact yolks*

The data from the three separate experiments were essentially the same. They have been combined and are presented in Table 3. The results clearly show that invasion of the contents of intact yolks by *S. enteritidis* is markedly influenced by the age of the eggs before inoculation. Thus, a high proportion of yolks from eggs held for 6 weeks before inoculation were salmonella-positive whereas those in fresher eggs remained largely salmonella-negative (Table 3).

As the data in Table 3 illustrate, from an inoculum of $\log_{10}$ 2·7 cells (500), the

Table 2. *The influence of egg age on the ability of* S. enteritidis *to grow in albumen either removed from the egg or next to the yolk*

Material inoculated	Log_{10} increase (±S.E.) in the number of salmonellas during a 5-day storage period post inoculation
Whole egg contents*	
†Stored eggs	7·13±0·44
Stored yolks with albumen from fresh eggs	7·45±0·52
†Fresh eggs	0·32±0·08
Fresh yolks with albumen from stored eggs	0·57±0·04
Separated albumen	
Albumen from fresh eggs	0·36±0·02
Albumen from around yolks of stored eggs	0·68±0·09
Albumen from away from yolks of stored eggs	0·28±0·01

* In whole egg contents, inoculum was placed in albumen next to the yolk.
† Fresh eggs were 1-day-old and stored eggs 6 weeks old.
Data are the mean values from three separate experiments.
In each experiment, 20–60 eggs were examined.

Table 3. *The impact of egg storage prior to inoculation on the ability of* Salmonella enteritidis *to invade the intact yolk in whole egg contents*

Parameter measured	Eggs stored for the following number of days before inoculation* 1	21	42
No. eggs with salmonella-positive yolks†	3	8	47
Mean log_{10} c.f.u./yolk±S.E. (Range)	3·0±0·9 (1·8–4·8)	6·1±1·2 (3·6–10·1)	9·9±0·2 (6·2–10·9)
Mean log_{10} c.f.u. in albumen±S.E. in eggs with salmonella-positive yolks (Range)	5·6±1·3 (4·3–8·2)	5·7±0·8 (3·3–8·6)	7·6±0·4 (5·2–9·7)
Mean log_{10} c.f.u. in albumen±S.E. in eggs with salmonella-negative yolks (Range)	3·8±0·07 (3·3–5·1)	4·2±0·2 (2·4–7·6)	4·9±0·4 (2·6–7·4)

* Eggs inoculated, near the yolk, with approximately 500 cells of *S. enteritidis*.
† 60 eggs examined.

albumen of fresh eggs is able to support a population of approximately log_{10} 4·0 cells of *S. enteritidis* in the absence of yolk invasion. Levels of contamination were higher in the albumen of older eggs and, occasionally, reached log_{10} 7·0 per egg without yolk invasion (Table 3). This, however was unusual and in the great majority of eggs containing more than log_{10} 5·0 cells the yolk was positive for *S. enteritidis* and usually contained more cells than the albumen (Table 3).

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DISCUSSION

The work presented in this paper largely concentrates upon factors influencing the growth of *S. enteritidis* in albumen around the intact yolk. Previous publications [8, 11] have demonstrated that this area appears to be an important site of contamination in the contents of intact eggs and that growth of the bacterium occurs as a result of storage-related changes in the egg [8]. Experiments were designed to investigate the impact of egg storage conditions on the ability of separated albumen and whole egg contents to support the growth of *S. enteritidis*. A comparison of growth patterns in these materials made it possible to identify the areas within an egg most affected by storage.

The results of the various investigations confirm previous observations [8] that egg age has a profound effect on the multiplication of *S. enteritidis* in the albumen of whole egg contents. Growth was often rapid in 'stored' eggs, but little or no growth took place in albumen next to 'fresh' yolks (Fig. 1, Tables 1–3). These results could imply that albumen becomes less inhibitory to salmonellas during storage. This does not seem to be the case. Thus, when albumen from 'fresh' eggs was replaced with that from eggs that had been stored for 6 weeks at 20 °C no significant growth occurred (Table 2). This observation could also indicate that there is no significant leakage of material from yolk contents into albumen during storage; a view substantiated by the fact that little or no growth took place in albumen taken from around the yolks of eggs stored for up to 6 weeks (Table 2). The multiplication of *S. enteritidis* in albumen around the yolks of 'stored' eggs was often very rapid and large populations could be achieved (Tables 1–3). Growth was unaffected by the age of the albumen and was equally rapid when that from 'stored' eggs was replaced with material from eggs that were 1 day old (Table 2).

Egg albumen is a dynamic system and research [18] has shown that, with storage, there is a relative increase in the volume of outer thin white, and a decrease in the thin and thick white surrounding the yolk. There is thus the possibility that these changes might have influenced the sampling of albumen. Results from any experiments using artificial contamination must be interpreted with caution. However, the high level of agreement between experiments and the small standard error within individual experiments suggests that the observations reported in this paper are unlikely to be only artifactual.

The potential problem of mixing of albumen was considered during the design of experiments where material was interchanged between groups of eggs. It was felt that the breaking of fresh eggs into sterile containers which were then left untouched until testing coupled with the removal of albumen only from around the yolk would minimize the dilution of material which may have come from yolk contents and localized only around the yolk.

The results presented in this paper suggest that storage has little direct impact on albumen with respect to the growth of salmonellas. The data could be taken to indicate, however, that changes are taking place to the integrity of the vitelline (yolk) membrane. Fromm [17] demonstrated that, during storage, the weight and protein and hexosamine content of the vitelline membrane declined. These changes may result in the membrane being more permeable. Although the data on albumen (Table 2) may suggest that increased permeability does not result in the

significant escape of material from yolk contents it would appear to allow *S. enteritidis* to invade more easily (Table 3). This will result in eggs containing a substantial population of salmonellas. This is of clear public health importance and warrants further investigation. The vitelline membrane is an acellular multi-layered tissue and as Burley and Vadehra [16] conclude 'the precise role of the membrane in the transport of molecules between the albumen and the yolk has still to be determined'.

As stated earlier, the albumen around the yolk would appear to be an important site for contamination with *S. enteritidis* [8, 11]. In this position the growth parameters of the bacterium seem to be divided into three distinct phases. During the first 24 h after lay, when the pH of the albumen is rising to approximately pH 9·2, there is multiplication of the bacterium and an approximate tenfold increase in the number of salmonellas. Thereafter there is a slow increase in the growth rate (Fig. 1, Table 1) which is not significant. During this second phase, *S. enteritidis* is still confined to the albumen even when the bacterium was inoculated onto the outside of the vitelline membrane. The final phase of rapid growth, where high populations ($> \text{Log}_{10}$ 10·0 per egg) of salmonellas can be achieved within the 5-day incubation period, would appear to be associated with invasion of yolk contents although substantial populations can also be present in the albumen (Table 3). Thus *S. enteritidis* behaves in essentially the same manner in egg contents as rot-producing bacteria [1]. There is one important difference, however. Even in eggs carrying high populations ($> \log_{10}$ 6·0 per egg) of salmonellas it was not possible to detect their presence on cursory examination and apart from faint turbidity in some areas of albumen there were no visible signs of contamination. This is in line with previous observations on other salmonellas [2, 4].

The speed with which the postulated changes in membrane integrity take place during storage is strongly temperature dependent. At 20 °C no significant changes occurred over 3 weeks, whereas after only 7–10 days storage under conditions where temperatures fluctuated between 18 and 30 °C, *S. enteritidis* was able to grow rapidly (Fig. 1).

In the United Kingdom, eggs are not generally refrigerated at point of sale. Major supermarkets attempt to maintain store temperatures at 19–20 °C. Provided that these temperatures are not exceeded, the results with naturally contaminated eggs [8], supported by work presented in this paper, would suggest that refrigerated storage in retail outlets might not be necessary but that the shelf-life from lay should be no longer than 21 days. The limited survey of small shops referred to earlier revealed that in the summer months eggs can be displayed, in some establishments, at above 30 °C. This may have important consequences for the public health. If retailers have difficulty in maintaining shop temperatures at or around 20 °C consideration should be given to refrigerated storage of eggs. This can also be used to extend shelf-life. Temperatures can also fluctuate markedly in the kitchen and consumers should continue to observe the advice issued by the United Kingdom Chief Medical Officer [19] that, in the home, eggs should be stored under refrigeration.

The low frequency of contamination with eggs from naturally infected birds [8] meant that it was necessary to use artificial methods of contamination. The authors recognize that caution should be exercised in the extrapolation of results

from this study to what may take place with natural contamination. However, the close correlation between the results with eggs held at 20 °C and earlier work with naturally contaminated eggs stored at that temperature [8] suggests that the data presented in this report may provide an insight into factors controlling the growth of *S. enteritidis* in egg contents.

Iron limitation has been shown to be important in controlling the growth of salmonellas in egg albumen [15]. A detailed examination of changes in the availability of iron was outside the scope of the studies reported in this paper. There is the strong possibility that the large amounts of iron in yolk contents become available to *S. enteritidis* as a result of storage-related changes to the vitelline membrane. Direct access, as a result of yolk invasion, would seem to be most important but, particularly in eggs where a large population of *S. enteritidis* is confined to the albumen, there is also the possibility that iron-binding siderophones may be able to cross the altered vitelline membrane [20].

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