The identification of the optimum conditions for the embryonation of *Toxocara canis* ova

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Suspensions of unembryonated *Toxocara canis* ova were maintained at 24°C, 30°C and 37°C in solutions of 0.4% and 1.6% and 3% formalin. At temperatures between 24°C and 30°C an increase in the rate of embryonation was observed especially from the morula to the early motile embryo stages. At 37°C, embryogenesis failed to develop. The rate of embryogenesis was found to be slower the higher the fixative concentration became. Over time, a decline in the percentage viabilities of the embryonated ova was also found as the fixative concentration increased. It seems that the fastest means to produce embryonated ova is to maintain them in a solution of 0.4% formalin at 30°C and exposed to light. However, after this process, in order to ensure the long term survival of these ova, it appears that it is best to store them at 24°C or less and ensure that a sufficient concentration of dissolved oxygen is available.

**Keywords:** *Toxocara*; embryonation; temperature; formalin; oxygen; light.

**Introduction**

The widespread contamination of the environment of *Toxocara canis* ova by dogs, especially in public areas such as parks and playgrounds, can lead to infection in children (Glickman and Schantz 1981). For these ova to be infective however, they must undergo a process of embryonation but the tough resistant shell of *T. canis* ova can mean that they can survive for many years in soils especially those with a high moisture content (Glickman and Schantz 1981). To date, no standardised criteria as to how best to create the ideal conditions under which *T. canis* ova can become embryonated have been established. The benefit of such could mean that the problem of acquiring and isolating sufficient numbers of viable embryonated ova from either faeces or whole worms would be greatly reduced once these ideal conditions were identified particularly as the absence of commercially available *T. canis* excretory-secretory larval antigens means that the *in vitro* culture of hatched second stage (L₂) larvae is often necessary for the routine sero-epidemiological testing of humans.

**Materials and methods**

Adult *Toxocara* worms were removed from stray dogs on the day they were euthanised so that all worms found were still alive prior to their fixation in 1% formalin solution. The uteri from these nematodes were carefully removed intact and only those segments containing viable unembryonated ova were selected for experimental work. To prevent
premature embryonation from occurring, the uteri and ova were kept on ice during all stages of the dissection process of worms. Mature ova, at the tapering end of the uterus (near the vulva at the anterior end of the worm) were identified microscopically at $\times 400$. The characteristic pitted surface design of the egg shells and the uniformity of the cellular contents were the two most important criteria used for the selection purposes.

An embryonation experiment was designed to observe the influence of temperature and fixative (formaldehyde) concentration on the development of larvae within their egg shells. Suspensions of unembryonated *T. canis* ova were maintained at 24°C, 30°C and 37°C in 0.4% and 1.6% and 3% formalin solutions in plastic Sterilin® containers. Internal light sources were found to be unsuitable in incubators as they emitted excessive amounts of heat, so external light sources were used instead. Desk lamps were used for this purpose and these were situated at various distances from the incubators which were equipped with glass panels. This meant that the margin of error with respect to the internal temperature would be $\pm 2^\circ\text{C}$ at most.

According to Onorato (1931), one of the benefits of storing ova in weak formalin solutions was to keep down bacterial growth since previous work had shown that the growth of bacteria prevented embryonic larval development by using up the oxygen in the suspension. However, this was also the reason why a control suspension, i.e. one without formaldehyde, was not used as part of this embryonation experiment. Such a control suspension would have been useful in assessing the importance of oxygen concentration and its gradual depletion by the embryonated ova over time.

Otto (1929) observed that all ova do not develop uniformly within a suspension. So, in order to graph the entire development over time, the several stages within a suspension were expressed as a single factor of development. This is why an index of development system similar to that which was devised by both Otto (1929) and later by Onorato (1931) was used for this study. Each stage of the embryonation process was assigned an index value as follows: a viable unembryonated single celled state $= 1$; a 2 or 4 cellular divided state $= 2$; an early morula state $= 3$; a late morular/early tadpole state $= 4$; a tadpole state $= 5$; a late tadpole state $= 6$; a motile embryo state $= 7$; an infective larval state $= 8$; a degenerate state $= 0$. About the same length of time is taken in developing from one of the listed stages to the next, hence a simple arithmetical series was used to represent the stages. With this system, the percentage of ova in each stage was multiplied by the factor for that stage and the sum of these factors was then the total factor of development for the suspension under consideration. If all the ova were embryonated then the factor of development was 800.

One problem did emerge which was the ever present source of error in determining whether or not an egg was viable. No attempt was made to determine mobility of the late embryonic stages. In addition, the percentage of embryonated ova present in a suspension at any given time could not be deduced from the index of development value alone. The process of embryonation in each suspension was monitored once each day for the first crucial 9 days and then every 3 days thereafter until day 21 and then finally on day 29. As time is such a critical factor, particularly in the early stages of embryogenesis, readings were recorded in minutes rather than in days or hours.

Using the Data Desk package, Chi-square tests were performed on the numbers of viable and non-viable embryonated ova present in suspensions maintained under various conditions of fixative concentration and temperature over time. Significance was defined as $p \leq 0.05$. 

Results

Increasing the temperature was found to increase the initial rate of embryonation. At 37°C, the embryonation process went no further than the morula stage but no morphological disintegration was apparent however until after approximately 13000 min (i.e. 220 h or 9 days). It would seem therefore that 30°C is the optimum temperature and 37°C the least favourable. It was found that the higher the temperature, the higher the initial rate of development, particularly from the morula to the early motile embryo stages (Figs 1–3). In order to highlight the effects of temperature and fixative concentration on the embryonation process, the time scales in the figures have been arranged to exclude those index values recorded after a time of 10000 min (i.e. 166 h or 6.9 days) when the process of embryonation approaches completion and the index values level off.

It was also observed that as the fixative concentration increased, the viability of the

![Fig. 1. The index development values for T. canis ova suspensions maintained in 0.4%, 1.6% and 3% formalin solutions at 24°C.](image)

![Fig. 2. The index development values for T. canis ova suspensions maintained in 0.4%, 1.6% and 3% formalin solutions at 30°C.](image)
embryonated ova declined somewhat at 24°C and especially at 30°C (Table 1). Chi-square analysis of the actual numbers of viable and non-viable embryonated ova under the different fixative concentrations was also found to be significant at both 24°C ($\chi^2 = 14.68; \text{df} = 2; p \leq 0.0006$ and 30°C ($\chi^2 = 49.80; \text{df} = 2; p \leq 0.0001$). Evidence was found of a decline in the percentage of embryonated ova present in the various suspensions when examined on days 15, 21 and 29 (Table 1). The difference in the numbers of viable and non-viable embryonated ova during this time was also statistically significant at 30°C ($\chi^2 = 13.18; \text{df} = 2; p \leq 0.0014$) but not at 24°C ($\chi^2 = 0.4661; \text{df} = 2; p \leq 0.7921$). When the relationship between temperature and the viability of the embryonated ova was examined, the result was statistically significant ($\chi^2 = 10.32; \text{df} = 1; p \leq 0.0013$).

**Discussion**

No embryonation occurred in *T. canis* ova kept at 37°C although the late morula stages were reached. Similar findings were also observed by Onorato (1931) and Okoshi and

**Table 1.** The percentage of viable *T. canis* embryonated ova present in suspensions of 0.4%, 1.6% and 3% formalin solutions at both 24°C and 30°C. Each observation is the result of a single reading taken 15, 21 and 29 days after the process of embryonation has commenced.

<table>
<thead>
<tr>
<th>Temperature% fixative conc.</th>
<th>24°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>% fixative conc.</td>
<td>Day 15</td>
<td>Day 21</td>
</tr>
<tr>
<td>0.4</td>
<td>98.63</td>
<td>98.09</td>
</tr>
<tr>
<td>1.6</td>
<td>97.78</td>
<td>92.31</td>
</tr>
<tr>
<td>3.0</td>
<td>94.14</td>
<td>97.77</td>
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Usui (1968) where the ova subsequently underwent degeneration. In contrast, Cuellar del Hoyo et al. (1986) found that embryonation was complete after 32 days when ova were incubated in sterile saline at 37°C but only when exposed to light. The high rate of mortality in suspensions maintained at 37°C in the present study might suggest that a change in the structural composition of the egg shell occurs making it permeable to fixative. The death of the developing embryos at 37°C is perhaps the result of the toxic effect of fixative rather than the high temperature even though the concentration of oxygen in the solution is likely to be less than that at lower, cooler temperatures. The use of a control would have clearly established which of these three factors is the most important in terms of egg viability and survival.

At both 24 and 30°C, the percentage proportion of the suspensions with fully embryonated ova generally exceeded 90% after only 9 days in the present study. In Okoshi and Usui's study (1968), 98% of the ova developed into complete embryos (second stage L2 larvae) in 11 days at 30°C, and at 25°C in 14 days. According to Onorato (1931), embryonation was complete in some but not in all ova after 7 days at 24°C and at 30°C after 4 days in water. The latter observation was interpreted by the authors as evidence that 30°C was the optimum condition under which ova developed the fastest. Certainly the results from the present study would suggest that this is in fact the case.

Cuellar del Hoyo et al. (1986) reported that the time it takes for embryonation to be complete is independent of the incubation medium. This finding contrasts with the results of the present study especially at 30°C and in 3% formalin solution (Figs 1 and 2, Table 1). It appears that the higher the fixative concentration the greater the mortality rate among the embryonating ova. There is also evidence that once embryogenesis is complete, the percentage of embryonated ova declines over time with increasing concentration of fixative (Table 1). Of course, this may be partly due to a decline in the level of dissolved oxygen in the incubation medium over time rather than due to the effect of fixative alone.

Conclusions

It appears that the fastest means to produce embryonated ova is to maintain them in a solution of 0.4% formalin (or one with a lower concentration) at 30°C when exposed to light and then to store these ova at 24°C (or at a lower temperature) in order to maintain their viability, ensuring that there is a sufficient concentration of dissolved oxygen present. Given the optimum conditions with which *T. canis* ova are known to survive, it would seem that any widespread environmental contamination could lead to a long-term health impact especially in a mild temperate climate where high levels of moisture in the soil exist. Although *T. canis* ova are vulnerable to dessication, their ability to withstand the effects of formalin solutions should be an important consideration in any sanitary programme to chemically treat contaminated soils in public places.

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References


