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Research paper

Survival of *Taenia saginata* eggs under different environmental conditions

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ABSTRACT

This study aimed to assess *in vitro* the transmission potential of *T. saginata* eggs stored in different media at various temperatures, and to optimize recovery, hatching and activation methods. A total of 42 freshwater and 42 silt samples were spiked with 100 µl of a *T. saginata* egg suspension, of which half were stored at 5 °C and the other half at 20 °C. Additionally, 5 silt and 35 tap water control samples were included. Eggs were obtained from gravid proglottids passed by a human carrier following treatment. The duration of the experiment was 6 months, with three sampling time points at 2, 4 and 6 months, respectively (Study 1). In addition, two pilot studies were carried out. One included 8 samples kept in a freshwater stream for a period of 2 and 4 months, respectively, from December 2016 until February 2017 (Study 2). Another study used 6 water samples which were stored for one week in the freezer at −18 °C, and 6 samples that were stored outdoor from 7 to 14 February, at temperatures ranging from − 6 °C to 5 °C (Study 3). To assess survival of *T. saginata* eggs, recovery, hatching and activation methods were optimized. *Taenia saginata* eggs could be activated after 6 months of storage in water and silt at 5 °C. Storage at 20 °C significantly decreased activation of the eggs, to 4 months when stored in water and 2 months when stored in silt. Furthermore, degenerative changes in oncospheres were observed when eggs were stored at 20 °C, which were associated with an increased loss of oncospheres during activation. Eggs could be activated after 4 months of storage in the stream at temperatures ranging from - 10 °C to 17 °C, as well as after one week of constant freezing at – 18 °C, or repeated freezing and thawing from – 6 °C to 5 °C. This study indicates that *T. saginata* eggs can survive a Northern European winter, and thus pose a significant risk of transmission, and that *in vitro* activation is a more accurate method for assessing the transmission potential of *T. saginata* eggs, than recovery, integrity and hatching. Studies will be needed to assess how accurate the *in vitro* activation correlates to *in vivo* infectivity to ensure an accurate assessment of the transmission potential at a larger scale for surveillance purposes.

1. Introduction

Taenia saginata is a zoonotic parasite causing taeniosis in humans and bovine cysticercosis. Humans host the adult tapeworm in the small intestine and excrete proglottids containing *T. saginata* eggs with faeces (Dorny and Praet, 2007). According to King and Fairley (2015), the tapeworm releases from thousands to millions of eggs into the environment every day by an infected human. Cattle become infected by accidentally ingesting viable eggs from contaminated water sources, pastures or feed and humans become infected by eating raw or undercooked beef containing viable cysticerci. Laranjo-González et al. (2016) estimated that in Europe 11 million people suffer from taeniosis caused by *T. saginata*. Currently, the surveillance system for bovine cysticercosis in Europe is based on routine meat inspection and management of infected carcasses but neither bovine cysticercosis nor human taeniosis are notifiable according to EU regulations. While specific environment-oriented control measures are currently not implemented, *T. saginata* eggs have been found to survive in the environment for several months (Jepsen and Roth, 1952; Ilsøe et al., 1990). Water represents a major way of dissemination of *T. saginata* eggs, which have been detected in water bodies where treated or untreated sewage effluents were discarded (Kyvsgaard et al., 1991; Rossi et al., 2015; Cabaret et al., 2002; Boone et al., 2007).

To assess *T. saginata* egg survival, no standardised *in vitro* method

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exists, but several authors have used oncosphere activation, *i.e.* the ability of the embryo to demonstrate motility, and to actively escape the oncospheral membrane (Silverman, 1954a; Stevenson, 1983; Kyngdon et al., 2006; Mendlovic et al., 2014). Prior to activation, the oncosphere must escape the enclosing embryophore, a process called hatching. However, the term hatching has been used to describe both the disintegration of the embryophore and the activation of the embryo (Lucker, 1960; Jones et al., 1960; Stevenson, 1983). This study aimed to assess the survival of *T. saginata* eggs stored in different media at various temperatures, and to optimize and compare recovery, hatching and activation methods.

2. Materials and methods

The egg suspensions used in these experiments were received from the Institute of Tropical Medicine, Belgium. The eggs were obtained from fresh gravid proglottids of *T. saginata*, collected from a human carrier following treatment. Species determination was done by Polymerase Chain Reaction followed by Restricted Fragment Length Polymorphism (PCR-RFLP) on a mitochondrial 12S rDNA fragment (Geysen et al., 2007). The proglottids were washed in buffered phosphate saline (PBS) and the eggs were manually extracted. Penicillin 100 IU/ml, streptomycin 100 µg/ml and amphotericin B 0.25 µg/ml were added to the egg suspension in PBS (14 ml), which was then stored at 4 °C for 9 days prior to shipment. The material was in transit for 3 days. Upon arrival, the egg suspension was stored at 5 °C for 1 month until the main experimental study started.

Two batches of eggs were used, one for the initial experiments of recovery and one for the main experiment. The egg suspension concentration was determined by microscopic examination at 100x magnification of 10 µl aliquots. The quantification of 10 replicates revealed an average concentration of 65 eggs/10 µl egg suspension and therefore, the egg suspension used in the main experiment was estimated to contain a total of 89,700 *T. saginata* eggs.

Freshwater and silt were collected from a water stream located North of Copenhagen, Denmark. In the laboratory, silt was sieved with a 1000 µm sieve to remove large particles, and both freshwater and silt were sterilized at 110 °C for 30 min.

2.1. Study design

For Study 1, *Taenia saginata* eggs were incubated at 5 °C and 20 °C with 7 replicates in both freshwater and silt, for 2, 4 and 6 months, respectively. Apart from freshwater and silt, *T. saginata* eggs were also stored in tap water as controls with 5 replicates at both temperatures for six months.

In total, 42 freshwater and 47 silt samples were prepared, as well as 35 tap water controls. Each silt sample was prepared by placing 5 g of silt and freshwater up to 20 ml in a 50 ml falcon tube, and each water sample contained 14 ml water in a 15 ml tube. Each sample was spiked with 100 µl of the egg suspension.

At each time point for each replicate, recovery, integrity, hatchability and activation were measured. The counts from tap water and silt controls at day 0 were assumed to be the same at 5 °C and 20 °C; therefore, no replicates for 20 °C were examined on day 0. Furthermore, the counts from tap water at day 0 were assumed to be the same for freshwater at day 0, hence no replicates for freshwater at day 0 were examined.

Two smaller pilot studies were conducted as well. In Study 2, *T. saginata* eggs were confined in small plastic tubes of 3 cm length and 1 cm diameter, sealed at both ends with 20 µm nylon mesh. The tubes were confined in a plastic bottle with several holes and the bottle was placed in a water stream North of Copenhagen from the 22nd December 2016 until 21st April 2017. Four replicates, each spiked with 100 µl of the egg suspension, were prepared and kept for 2 and 4 months, respectively.

In Study 3, 6 replicates of *T. saginata* eggs in tap water were stored for one week in the freezer at – 18 °C and outdoor from 7 to 14 February 2017 at temperatures ranging from – 6 to 5 °C. All samples were stored in 15 ml falcon tubes containing 14 ml tap water and spiked with 50 µl of the egg suspension. The outdoor temperature was measured with an electronic minimum/maximum thermometer.

2.2. Survival assessment of T. saginata eggs

In order to assess the survival of the eggs *in vitro*, they first needed to be recovered from silt and water. During the quantification of the recovered eggs, the integrity of the embryophore was also examined. Based on general appearance, only eggs with an intact and thick embryophore were considered for viability assessment. Afterwards, the eggs were hatched and the resulted oncospheres as well as non-hatched eggs were quantified. The final step was the activation of the oncospheres, followed by examination and quantification of the activated and non-activated oncospheres. A viable oncosphere was defined as one that can be activated *in vitro* to show embryo motility and/or secretory globules.

All the examinations and quantifications were conducted microscopically with 100× magnification under an Olympus Bx40 microscope. To ensure a reliable assessment of viability, the entire final sample volume (1 ml) was assessed, in a glass Sedgewick rafter counting chamber. Furthermore, the pH was measured for each replicate prior to egg examination with a microprocessor-based pH and temperature bench meter (Hanna instruments Inc.).

2.3. Recovery of T. saginata eggs from water samples

To recover eggs from water samples, the 15 ml tubes were centrifuged at 253 x g for 7 min in a bench top centrifuge (Scanspeed 1236), and the supernatant was removed with a pipette leaving 1 ml of sediment containing the eggs. The sediment was transferred into the counting chamber and the eggs quantified at 100× magnification. In the case of the water stream pilot study, the tubes were transported to the lab in a bottle filled with fresh water, which was stored at 5 °C upon arrival. Each tube was opened at one end and the content of the tube was washed into a 15 ml tube. In the case of the freezing pilot study, the frozen water samples were thawed in the refrigerator at 5 °C before examination. The subsequent steps were identical as described above.

2.4. Recovery of T. saginata eggs from silt

The recovery method of choice was adapted from a technique for isolation of parasite eggs from soil, mainly ascarid eggs (Larsen and Roepstorff, 1999). The efficiency of the egg-recovery technique was assessed by adding known numbers of *T. saginata* eggs to the silt samples.

Each silt sample was homogenized by mixing with a stainless-steel spatula. To dissociate the eggs from the silt particles in the sample, 37.5 µl Tween 80 were added to the spiked sample and mixed well with the spatula for 5 min. Afterwards, the sample was centrifuged at 253 × *g* for 7 min. The supernatant was removed and the sediment containing the eggs was resuspended in $ZnSO_4$ flotation solution, s. g. 1.350, up to a total volume of 50 ml. The flotation solution was obtained from 99.5% pure salt of $ZnSO_4$ heptahydrate ($ZnSO_4.7H_2O$), mixed with water in equal quantities, according to the Flotac manual (Cringoli et al., 2010). The sample was mixed well with the spatula, then centrifuged at 253 × *g* for 7 min. The supernatant containing the eggs was poured into a beaker. The sediment was resuspended in flotation solution up to a total volume of 50 ml and centrifuged again at 253 × *g* for 7 min, after which the supernatant was poured in the same beaker.

For sieving, a stack of 3 Endecotts sieves was used, with a diameter of 20 cm and mesh sizes of 212, 106 and 20 µm. The sediment was washed through the sieves to remove the flotation fluid from the eggs,

while shaking the sieves continuously to remove excess water. The material from the 212 and 106 µm sieves was discarded. The material collected in the 20 µm sieve was washed into a 50 ml falcon tube and centrifuged at 253 × *g* for 7 min. The supernatant was removed with a pipette. The sediment was poured into the counting chamber, followed by microscopic examination.

2.5. Hatching of T. saginata eggs

Sodium hypochlorite was chosen as the hatching medium for *T. saginata* eggs using a commercial 2.7% sodium hypochlorite, in a 2/6.5 dilution in water at 37 °C (2 ml sodium hypochlorite added to 6.5 ml water at 37 °C).

One ml of the freshly prepared hatching solution was mixed with 1 ml egg suspension and the tube was shaken continuously by hand for 6 min. During this time, the embryophoric blocks disaggregated from the majority of the eggs. After the 6 min, water was added up to 14 ml and the suspension was centrifuged at 253 × *g* for 7 min. The oncospheres were washed 4 times by centrifugation in water. The oncospheres were sedimented by centrifugation and the supernatant was removed leaving 1 ml of the concentrated oncosphere suspension, which was transferred to a counting chamber, followed by examination at 100x magnification. The hatched and not hatched eggs were counted. After counting, the suspension was washed in a tube and centrifuged at 253 × *g* for 7 min.

2.6. Activation of T. saginata eggs

Taenia saginata oncospheres were activated by incubation in 2 ml of an artificial intestinal fluid (AIF) solution of 1% pancreatin, 1% $NaHCO_3$, 1.6% bile and 0.04% trypsin in water up to 2 ml, for 45 min in a 37 °C water bath. Fresh bile was collected at slaughter from the gall bladder of a healthy steer and was stored at −20 °C until use. All in powder form, trypsin and pancreatin were stored at −20 °C until use, while $NaHCO_3$ was stored at room temperature.

The AIF solution was filter sterilized before use, using a 20 µm syringe filter. The tube containing the oncospheres was removed from the water bath and shaken by hand for 1 min after each 5 min of incubation. After 45 min of incubation in AIF at 37 °C, the oncospheres were washed 3 times in RPMI at 37 °C, by centrifugation at 253 × *g* for 7 min. After centrifugation, the supernatant was removed leaving 1 ml of the oncosphere suspension, which was transferred to a counting chamber, followed by examination at 100x magnification. Oncospheres were considered to be activated if they presented motility and/or secretory globule, and both activated and not activated oncospheres were counted.

2.7. Data analysis

All statistical analyses were done using STATA 14.1 (StataCorp, College Station, Texas, USA). Comparisons between matrices (water/silt) and incubation temperatures (5 °C/20 °C) were done with non-parametric Mann-Whitney U-tests, using p-values below 0.05 as accepted level of significance. The statistical significance of the trends of recovery, integrity, hatching and activation over time was determined using an extension of the Mann-Whitney test for trend across ordered groups using the nptrend command. Median values of the counts were used to express the decrease in proportions of recovery, integrity, hatching and activation over time. To determine the proportions of the recovered eggs that were intact, hatched and active, three new variables were generated for analysis, representing the ratios of integrity, hatching and activation relative to recovery, which were subsequently compared to the recovery at each time point. Comparisons between media with respect to integrity, hatching and activation ratios were done for each of the time and temperature combinations.

3. Results

3.1. Initial studies of recovery

Initial analysis of the egg suspension by the count of 10 replicates of 10 µl revealed a median concentration of 65 eggs/10 µl. Although the egg suspension was mixed well before each sampling point, and the pipette was calibrated before use, the concentration varied from minimum 43 to maximum 84 eggs per 10 µl.

3.2. Comparison of the recovery at time point 0 between silt and water (Study 1)

With each sample being spiked with 100 µl of the egg suspension, the recovery from tap water at day 0 varied from 572 to 686 eggs (median: 631), and served as unexposed control for the treated water groups. At day 0, recovery from silt varied from 318 to 476 eggs (median: 403), and was used as unexposed control for the treated silt groups. Comparing the recovery at time point 0 between silt and water, there was a significantly lower recovery from silt compared to water ($p = 0.009$).

3.2.1. Recovery of T. saginata eggs over time (Study 1)

As illustrated in Fig. 1, a significant decrease in egg recovery over time was found from either tap water, freshwater and silt at both 5 °C ($p = 0.006$, $p < 0.001$ and $p < 0.001$, respectively) and 20 °C ($p = 0.001$, $p < 0.001$, and $p < 0.001$, respectively). An increase in temperature showed consistently lower recoveries, for each of the different media. After 6 months, there was a 16% decrease in recovery from tap water at 5 °C, and 25% decrease from tap water at 20 °C (median recovery: 532 and 472, respectively). Recovery from freshwater decreased with 19% after 6 months at 5 °C and with 29% at 20 °C (median recovery: 508 and 450, respectively). Over time, recovery from silt showed a more pronounced decrease, with a 41% reduction in recovery after 6 months at 5 °C and 50% reduction at 20 °C (median recovery: 238 and 202, respectively).

3.2.2. Integrity of T. saginata eggs over time (Study 1)

As shown in Fig. 1, egg integrity from tap water, freshwater and silt at 5 °C had a consistently decreasing trend over time ($p = 0.003$, $p < 0.001$ and $p < 0.001$, respectively), as was also the case for integrity from tap water, freshwater and silt at 20 °C ($p < 0.001$). As time increased, there were more irregular eggs (*e.g.* eggs with a broken shell or with what appeared to be air bubbles inside the oncospheres). After 6 months, integrity decreased by 20% in tap water at 5 °C and by 28% in tap water at 20 °C (median integrity: 517 and 450, respectively). In freshwater, integrity decreased with 21% after 6 months at 5 °C and with 31% at 20 °C (median integrity: 493 and 432, respectively). After 6 months, there was a 45% decrease in integrity from silt at 5 °C and 58% from silt at 20 °C (median integrity, 212 and 161, respectively).

3.2.3. Hatching of T. saginata eggs over time (Study 1)

Hatching from tap water, freshwater and silt had a significantly decreasing trend over time at both 5 °C ($p = 0.001$, $p < 0.001$ and $p < 0.001$, respectively) and 20 °C ($p < 0.001$), as shown in Fig. 1. After 6 months, there was a 15% decrease in hatching from tap water at 5 °C and 34% from tap water at 20 °C (median hatching: 498 and 385, respectively). After 6 months in freshwater at 5 °C hatching decreased with 25%, and at 20 °C with 38% (median hatching: 439 and 365, respectively). In silt, hatching decreased with 57% after 6 months at 5 °C, and with 76% at 20 °C (median hatching:157 and 87, respectively).

3.2.4. Activation of T. saginata eggs over time (Study 1)

As illustrated in Fig. 1, significant decreasing trends were seen in activation over time from tap water, freshwater and silt, at both 5 °C ($p < 0.001$) and 20 °C ($p < 0.001$). When looking at activation after 6

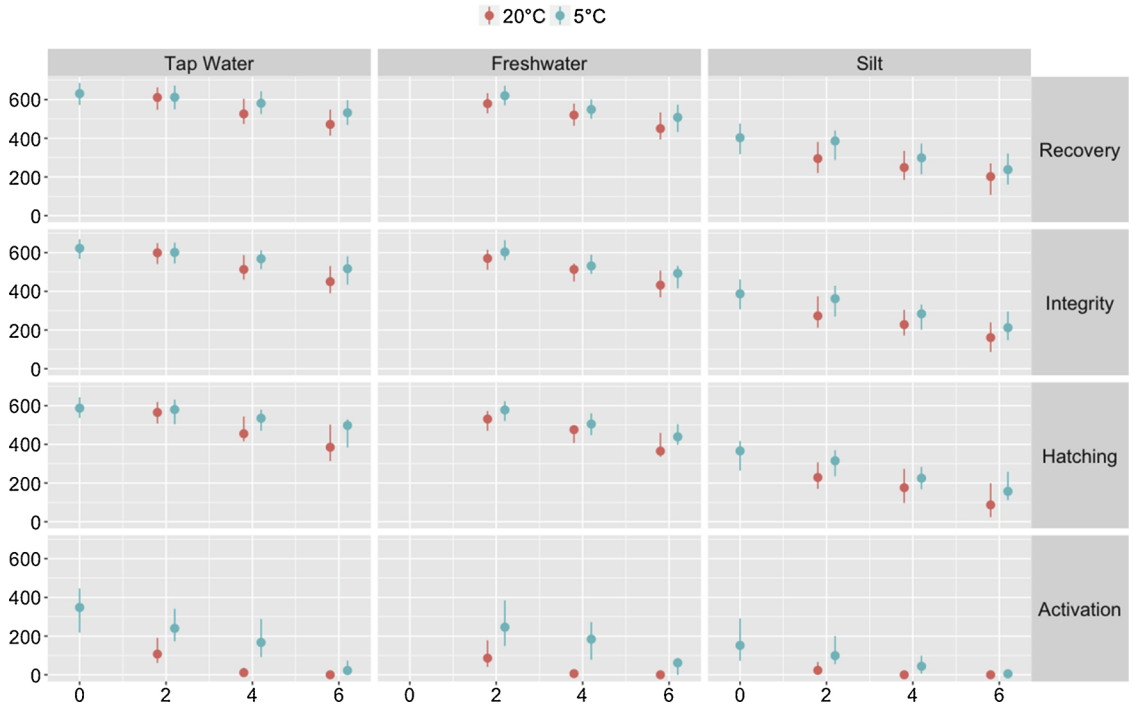

Fig. 1. *Taenia saginata* egg median recovery, integrity, hatching an activation from tap water, freshwater and silt, stored at either 5 °C or 20 °C. The red and blue lines represent the range of the values measured at each time point. The values from time point 0 are based on 5 replicas and the values from time points 2, 4 and 6 are based on 7 replicas.

months, a steeper decrease was observed, in contrast to recovery, integrity and hatching, which decreased more gradually over time. In tap water at 5 °C, there was a 94% decrease in activation after 6 months (median activation: 22). In freshwater at 5 °C, activation decreased with 82% after 6 months (median activation: 62). In silt, there was a 97% decrease in activation after 6 months at 5 °C (median activation: 5).

Comparing media at 20 °C over time, consistently lower recovery, integrity and hatchability counts were recorded from silt as compared to tap water and freshwater. However, when looking at activation from tap water, freshwater and silt after 6 months at 20 °C, there was no difference between media, since no oncospheres were activated from either media.

3.2.5. Survival of T. saginata eggs in tap water at different temperatures (Study 1)

The median recovery, integrity, hatching and activation from tap water after 2, 4 and 6 months were consistently lower at 20 °C than at 5 °C. There were significantly less activated eggs in tap water at 20 °C compared to 5 °C after 2, 4 and 6 months ($p < 0.02$, $p = 0.009$, and $p = 0.005$, respectively). However, statistically, the difference between recovery, integrity and hatching from tap water at 5 °C and 20 °C after 2, 4, and 6 months, respectively was not found to differ significantly ($p > 0.05$).

3.2.6. Survival of T. saginata eggs in freshwater at different temperatures (Study 1)

The median recovery, integrity, hatching and activation from freshwater after 2, 4 and 6 months were consistently lower at 20 °C compared to 5 °C. However, statistically, the difference in recovery between freshwater at 5 °C and 20 °C after 2, 4, and 6 months was not significant ($p = 0.084$, $p = 0.225$ and $p = 0.142$, respectively). The difference in integrity from freshwater at 5 °C and 20 °C was found statistically significant after 2 months ($p = 0.048$), but not significant after 4 and 6 months ($p = 0.141$ and $p = 0.110$, respectively). The difference in hatching from freshwater at 5 °C and 20 °C was found statistically significant after 2 and 6 months ($p = 0.030$ and $p = 0.025$, respectively), but not significant after 4 months ($p = 0.073$).

Activation was significantly lower in freshwater at 20 °C compared to 5 °C after 2, 4 and 6 months ($p = 0.004$, $p = 0.002$ and $p = 0.003$, respectively).

3.2.7. Survival of T. saginata eggs in silt at different temperatures (Study 1)

The median recovery, integrity, hatching and activation from silt after 2, 4 and 6 months were consistently lower at 20 °C compared to 5 °C.

There were significantly less recovered, intact and hatched eggs in silt after 2 months at 20 °C compared to 5 °C ($p = 0.041$, $p = 0.048$, $p = 0.018$). However, statistically, no significant difference was found in recovery, integrity and hatching between 5 °C and 20 °C in silt after 4 months ($p = 0.306$, $p = 0.159$ and $p = 0.225$, respectively) and 6 months ($p = 0.180$, $p = 0.084$ and $p = 0.064$, respectively). Significantly less activated eggs were found in silt at 20 °C compared to 5 °C after 2, 4 and 6 months ($p = 0.006$, $p = 0.008$ and $p = 0.009$, respectively).

3.3. Activation proportions relative to the recovery at time point 0 (Study 1)

The median number of activated eggs decreased much faster if kept at 20 °C compared to samples kept at 5 °C. After 6 months, the median number of activated eggs relative to the median recovery at time 0 decreased to 3% in tap water, and 10% in freshwater at 5 °C, compared to 0% for samples kept at 20 °C. Likewise, the median number of activated eggs kept in silt decreased much faster than samples kept in water at the same temperature. After 4 months at 20 °C, the median number of activated eggs from silt relative to the median recovery at time 0 decreased to 0% and after 6 months at 5 °C, it decreased to 1%.

3.4. Pilot studies

3.4.1. Water stream experiment (Study 2)

The ambient air temperature in Northern Copenhagen area ranged between – 10 °C and 17 °C from 22nd December 2016 until 21st April

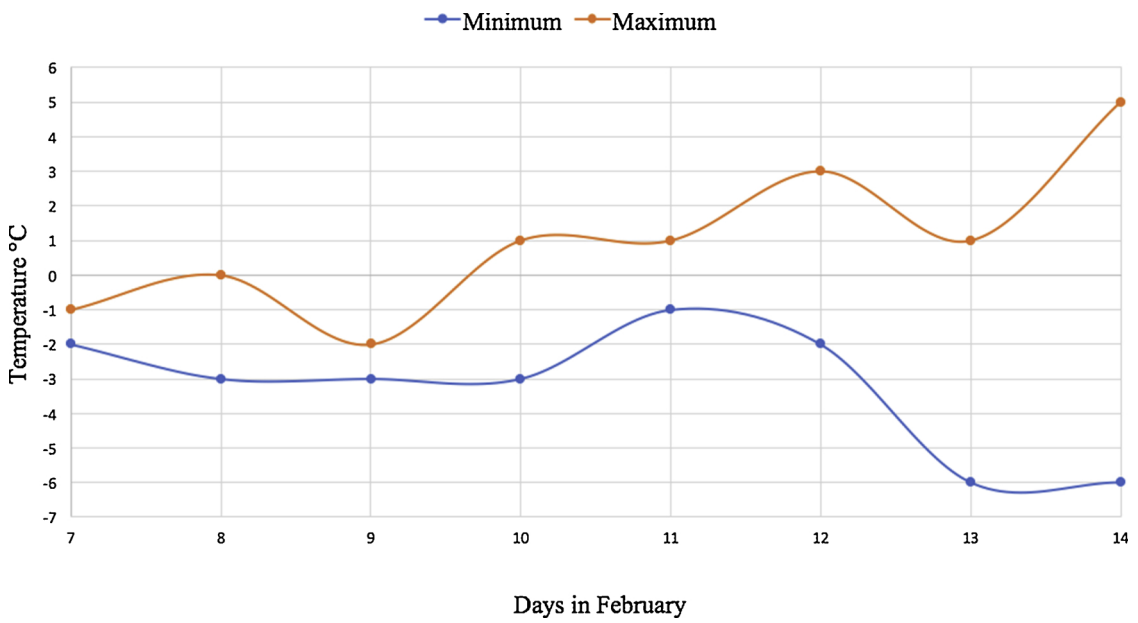

Fig. 2. Minimum-Maximum outdoor temperature in Copenhagen 7–14 February 2017.

2017. From 22nd December until 21st February, temperature ranged between – 10 °C and 11 °C (www.accuweather.com). The temperature of the water was not measured.

A decreasing trend over time was observed for recovery, integrity, hatching and activation. Recovery proportions are expressed relative to the total number of eggs seeded on day 0 of the experiment (650 eggs per replicate), whereas integrity, hatching and activation proportions are expressed relative to the recovery at each time point. After 2 months, the median recovery proportion was 57% (median: 372, range 277–496), whereas after 4 months, median recovery was 28% (median: 183, range 118–313). Integrity decreased from 89% after 2 months (median: 330, range: 260–461) to 73% after 4 months (median: 134, range: 89–239). Hatching decreased from 72% after 2 months (median: 269, range: 221–416) to 43% after 4 months (median: 79, range: 0–176). Activation decreased from 21% after 2 months (median: 79, range: 18–147) to 4% after 4 months (median: 8, range: 0–21).

3.4.2. *Freezing experiment (Study 3)*

Samples stored outdoor from 7th February until 14th February were exposed to temperatures ranging from – 6 °C to 5 °C, measured with a minimum/maximum outdoor thermometer (Fig. 2). Samples stored for the same period in the freezer were exposed to a constant temperature of – 18 °C. Consistently lower recovery, integrity and hatching, and greater variation among replicas were observed for the samples stored outdoor than for the samples stored in the freezer. Relative to the total number of eggs seeded in the beginning of the experiments (325 eggs per replicate), recovery proportions were 89% for samples stored outdoor (median: 288, range: 221–368), and 93% for samples stored in the freezer (median: 301, range: 274–331), respectively. Proportions of intact, hatched and activated eggs were expressed relative to the recovery for each group. Integrity proportion for the outdoor group was 89% (median: 256, range: 183–321), whereas integrity for the freezer group was 95% (median: 286, range: 249–325). Hatching proportions were 82% for the outdoor group (median: 235, range: 163–303), and 89% for the freezer group (median: 267, range: 230–301).

The activation proportions were very similar for the two groups. However, the variation was greater for the samples stored outdoor than for those stored in the freezer. An activation proportion of 33% was recorded for the outdoor group (median: 96, range: 38–154), and 32% for the freezer group (median: 96, range: 41–134).

4. Discussion

This *in vitro* study showed that *T. saginata* eggs could survive a Northern European winter, and thus pose a potentially significant risk of transmission. Storage for 6 months in tap water, freshwater and silt at 5 °C did not inactivate all eggs, whereas storage at 20 °C inactivated most eggs after 4 month when stored in water, and after 2 months when stored in silt. Moreover, degenerative changes in oncospheres were observed when eggs were stored at 20 °C, which were associated with an increased loss of oncospheres during activation. Exposure for 4 months at temperature fluctuations ranging from – 10 °C to 17 °C did not inactivate the eggs, and neither did one week of constant freezing at – 18 °C, or repeated freezing and thawing from – 5 °C to 6 °C. In addition, this study indicates that *in vitro* activation is a reliable indicator of egg survival and can be used to assess viability of *T. saginata* eggs. More studies are needed to investigate whether *in vitro* activation studies can replace *in vivo* experimental infection studies for the assessment of transmission potential of eggs.

Over time, recovery, hatching, integrity and activation of *T. saginata* eggs decreased significantly. Furthermore, recovery, integrity, hatching and activation were significantly lower for eggs stored at 20 °C than at 5 °C, regardless of media. However, the decrease in activation was steeper over time, particularly when eggs were stored at 20 °C, in contrast to recovery, integrity and hatching, which showed a moderate decrease over time. In this study, *T. saginata* eggs could be activated after 180 days of storage in tap water and freshwater at 5 °C. This was found to be within the range reported by two other studies that investigated the survival of *T. saginata* eggs *in vitro*, and reported a survival of 168 days in saline at 4–5 °C (Froyd, 1962), and 335 days in saline at 4 °C (Silverman, 1954a), respectively. Interestingly, when storing *T. saginata* eggs in saline at 20 °C, Silverman (1954a) found a survival of 60 days, whereas in this study, eggs in tap water and freshwater could be activated after 120 days at 20 °C. Assessment of egg survival in silt at different temperatures in relation to previous findings is limited by the fact that previous studies were conducted *in vivo* under field conditions, whereas this study was conducted *in vitro* under laboratory conditions. Nevertheless, the survival of 180 and 120 days found for eggs stored in silt at 5 °C and 20 °C, respectively, is comparable to the survival reported by Ilsøe et al. (1990), of 165 days on soil at temperatures ranging from 4 °C to 21 °C. However, in the study of Ilsøe et al. (1990), eggs were stored in nylon bags on soil, not as free eggs in

soil, therefore eggs did not have to be recovered from soil.

The demonstrated survival of *T. saginata* eggs stored in the water stream for 120 days at temperatures ranging from – 10 °C to 17 °C shows that eggs can withstand temperature fluctuations when stored in freshwater. The wide range in recovery of eggs may indicate that some eggs were disintegrated due to the temperature fluctuations, repeated thawing and freezing, and movement and pressure from the flowing water. Additionally, it was also demonstrated that one week of constant freezing at − 18 °C, and one week of repeated freezing and thawing from – 6 °C to 5 °C did not inactivate all eggs. However, repeated thawing and freezing was associated with a lower recovery. Previous studies indicated an *in vitro* survival of 90 days for eggs stored at –10 °C to 25 °C (Ilsøe et al., 1990), and 13–64 days for eggs stored at − 4 °C to 15 °C (Suvorov, 1965). However, it is not known what methods were used by Suvorov (1965) for *in vitro* survival assessment. Lucker (1960) reported an *in vivo* survival of 76 days for eggs stored at – 4.4 °C, although his results do not indicate maximum survival.

A decreased hatching over time for eggs stored at 20 °C was associated with a change in the general appearance of the hatched oncospheres after 4 months. The contents of the oncosphere appeared less clear, with a granular aspect, and the hooks were no longer visible, although the oncospheral membrane appeared intact. According to Scandrett and Gajadhar (2004) and Silverman (1954a), these observations were consistent with degenerative transformations of non-viable oncospheres. Moreover, Silverman (1954a) reported that dead oncospheres also became ovoid, as opposed to the characteristic egg-shape of viable oncospheres. It was indeed observed in this study while counting the hatched oncospheres that some had an ovoid shape while some had a round shape, but these observations were not quantified. The decrease in activation over time contrasted the findings of Jones et al. (1960), who reported that activation increased when eggs were stored. However, their findings were reported for eggs that were initially in different stages of development, whereas in this study, the majority of the eggs appeared well developed when extracted from the proglottid, with thick embryophores, which indicates the eggs were mature according to Silverman (1954b). Additionally, in this study, a decrease in activation over time was associated with an increase in the loss of oncospheres during the activation procedure, particularly at 20 °C. The degenerative aspects of the oncospheres when eggs stored at 20 °C were hatched appeared to be related to the loss of oncospheres during activation. This indicates that increased temperature accelerates the degenerative changes of oncospheres, in accordance with Havelaar and Block (1985). However, after 6 months, not only the samples stored at 20 °C, but also a large part of the samples stored at 5 °C showed oncospheres with degenerative appearance, and after the activation process, an increase in lost oncospheres was seen. Thus, there appears to be a correspondence between the general appearance of the oncospheres and their condition, in accordance with the findings of Silverman (1954a). As Stevenson (1983) reported, when eggs that were stored for a long period are hatched, a considerable proportion of oncospheres can be lost. Other factors might have contributed to the degeneration of the eggs, such as storage time and conditions before the start of the experiment. The egg suspension, already one month old when the experiment started, was in transit for three days, and eggs have probably been exposed to large temperature fluctuations. Furthermore, Stevenson (1983) demonstrated that eight times more oncospheres were lost when fluids containing trypsin were used for activation as compared to activation without trypsin, and argued that oncospheres were digested by trypsin. This effect of trypsin on degenerated oncospheres could explain the loss of oncospheres in this study. It is also possible that the permeability of the oncospheral membrane had changed over time, and the oncospheres were more readily digested by the activation fluid containing trypsin, as Stevenson (1983) suggested. The change in permeability was explored by studies on staining of the oncospheres. As Silverman (1954a) demonstrated, viable oncospheres maintain a semi-permeability of the oncospheral membrane and resist vital dies, whereas dead oncospheres readily absorb vital dies. This inability of the degenerated oncospheres to maintain that semi-permeability may explain the loss of oncospheres, by readily absorbing trypsin which simply digested their contents. Yet, in this experiment oncospheres failed to activate in fluids without trypsin, therefore trypsin was found essential for activation of *T. saginata* eggs. However, this consequence of using trypsin may be overcome by using calf serum, which could have a protective effect on the oncospheres and thus counteract this consequence of using trypsin, as Brandt and Sewell (1981) suggested. Furthermore, it is also possible that eggs that appeared intact might have had fine cracks within the embryophore which could not be detected with 100x magnification. Thus, during hatching, the sodium hypochlorite solution might have reached the oncospheres more readily and consequently damaged the oncospheres, as both Stevenson (1983) and Kyngdon et al. (2006) reported that sodium hypochlorite can quickly damage the oncospheres.

Significantly fewer eggs could be recovered from silt than from water at any time point. The variation among replicas could be related to the tendency of *T. saginata* eggs to cluster, as Silverman (1954b) showed that the fragile membrane surrounding the embryophore usually ruptures, leaving the yolk contents attached to the embryophore and causing the eggs to cluster. Furthermore, as Collender et al. (2015) described, eggs tend to adhere to solid particles and even to laboratory material, such as pipettes and tubes. However, assessment of these recovery proportions from tap water and silt in relation to earlier findings is difficult due to the lack of literature on recovery of taeniid eggs. Storey and Phillips (1982) reported a recovery efficiency of 84% for *T. saginata* eggs from soil drainage water, however, the technology used in that study was not available. Maya et al. (2006) recovered *T. solium* eggs from drinking water and wastewater effluent, however, the recovery efficiency of the method was not reported. Furthermore, over time a larger reduction in recovery was observed from silt than from water. Relative to recovery, integrity and hatching were significantly lower from silt than from water, which may indicate that the outer structures were more readily damaged in silt than in water, probably due to the complex procedures and chemicals used for the recovery of eggs from silt. However, no significant difference in activation was found between media at any time point, which indicates that the oncospheres were probably not affected by the recovery procedure.

In this study, survival of *T. saginata* eggs was assessed based on the ability of the eggs to show activity *in vitro*. However, to assess the survival of the eggs, they first had to be recovered from the environmental matrices. Although studies on recovery of taeniid eggs are lacking, several characteristics reported for taeniid eggs with emphasis on egg recovery from soils and bio solids have been taken into consideration in this study, which may have accounted for the satisfying recovery proportions from silt. Considering the tendency of the eggs to adhere to soil particles, a detergent was used to dissociate the eggs from soil particles, which was found to increase the egg recovery as compared to not using a dissociation agent. However, the composition of the silt might have influenced the recovery proportions as well, as Nunes et al. (1994) found the highest recovery of ascarid eggs from sand and the lowest from silty clay. In this study, the silt was composed of roughly two parts sand and one part clay. Soil particles, particularly clay may trap the eggs in the sediment, thus it was found essential to mix well the soil sample with the flotation solution to allow the eggs to float. Furthermore, the specific gravity of the flotation solution may also play a key role in recovery, as Collender et al. (2015) reported that the specific gravity of the flotation solution must be higher than the eggs. Although no specific gravity was reported for *T. saginata* eggs, a specific gravity ranging from 1.225 to 1.3 was reported for other taeniid eggs by David and Linquist (1982) and Maya et al. (2006). Initial studies on recovery with salt-sugar solution 1.270 s.g. and $MgSO_4$ solution 1.280 s.g. showed poor recoveries and it was therefore concluded that the specific gravity of *T. saginata* eggs must be higher than 1.280. Chacha et al. (2013) also found that when flotation solution with lower

specific gravity was used, isolation of taeniid eggs failed. Several studies used coverslips to recover eggs from flotation, however, with that technique, the eggs could not be used for any further tests. Other studies examined small aliquots from which they estimated the recovery proportions.

Hatching with sodium hypochlorite is a very quick and efficient method, as constantly high recovery proportions were seen within minutes, in agreement with the findings of Kyngdon et al. (2006) and Wang et al. (1997). However, it was reported that sodium hypochlorite could quickly damage the freed oncospheres, therefore controlled contact time with the right dilution and washing the oncospheres immediately after the embryophore disruption were essential (Stevenson, 1983; Kyngdon et al., 2006). Nevertheless, as Brandt and Sewell (1981) suggested, the sodium hypochlorite solution may need to be adjusted in accordance with the age and viability of the eggs.

According to Blagojevic et al. (2017), a new risk-based surveillance system suggested by EFSA in 2013 may be implemented in the future. Particularly developed to lower the potential risk of bacterial cross-contamination of the meat, the proposed system refers to reduced or no manual handling of the carcasses. However, given the already low sensitivity of the current meat inspection system for bovine cysticercosis, visual inspection only may not be sufficient to detect cysticerci. Therefore, in order to improve detection sensitivity, the new system would involve risk categorisation of the farm/animal and abattoir. As described by Blagojevic et al. (2017), risk classification of areas, farms and animals could be based on data and records from serological tests, history of bovine cysticercosis cases and other known risk factors on the farm, such as age, gender, production system, access to potentially contaminated pastures and water sources, and movement history (Calvo-Artavia et al., 2013; Laranjo-González et al., 2016). Based on this risk categorisation, it would be possible to perform visual-only inspection on low-risk cattle and detailed meat inspection on cattle that present a higher risk of infection (Laranjo-González et al., 2016; Blagojevic et al., 2017). Considering the recently proposed risk-based surveillance system, in order to risk-categorize farms and cattle, the way the disease spreads to cattle should be analyzed more closely. The risks associated with access to potentially contaminated pastures and water sources cannot be accurately assessed without considering the survival of *T. saginata* eggs in the environment and the potential for transmission. Therefore, more emphasis should be placed on the free-living stage of *T. saginata*. Activation of the embryo to show motility and to liberate itself from the enclosing oncospheral membrane is a confirmation that the embryo is alive and ready to invade the intestinal mucosa and develop into cysticerci if ingested by the intermediate host. *In vitro* egg survival can thus assess the transmission potential of the eggs. However, studies will be needed to assess how accurate the *in vitro* activation correlates to *in vivo* infectivity.

5. Conclusions

Taenia saginata eggs are robust, and can survive North European winters. Activation is a reliable *in vitro* indicator of egg survival, whereas recovery, integrity and hatching are not. Large proportions of intact eggs could be recovered and hatched, but significantly less could be activated. Furthermore, degenerative aspects of hatched oncospheres were associated with an increase in loss of oncospheres during activation and a decrease in activated oncospheres.

Knowledge on survival of the eggs is essential for a complete understanding of the transmission dynamics of *T. saginata*. Studies on abiotic and biotic factors affecting the *in vitro* survival of the egg could provide information on the number of eggs in the environment with transmission potential and thus replace *in vivo* studies. Therefore, *in vitro* egg survival and prevalence could serve as parameters to estimate the number of potentially infective eggs ingested by intermediate hosts and hence the risk of establishment of bovine cysticercosis. Furthermore, although not a serious public health threat, the economic burden of *T. saginata* should not be underestimated.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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