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Dispersal of taeniid eggs: Experimental faecal contamination of forest environment followed by DNA detection in wild berries

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ABSTRACT

To understand Taeniidae epidemiology, the principles of egg-dispersion dynamics under natural conditions must be known. In this study, non-zoonotic *Taenia laticollis* was used as a model parasite for the family Taeniidae (including *Echinococcus* spp.). An experiment to investigate dispersion from contaminated faeces to the surroundings was performed both with bilberries (*Vaccinium myrtillus*) and lingonberries (*Vaccinium vitis-idaea*), both of which are commercially harvested wild berries in Finland. For this experiment, 30 g of fox faeces was inoculated with 30,000 *T. laticollis* eggs for the bilberry experiment and 100,000 eggs for the lingonberry experiment. The faecal material was placed in the middle of good berry growth areas in four locations for bilberries and eight locations for lingonberries. After 41–42 days, berries at different distances (0–15 m) from the original contamination spot were collected and delivered to our laboratory. DNA was extracted from washed and sieved material and analysed using *T. laticollis*-specific semi-quantitative SYBR Green real-time polymerase chain reaction (qPCR). *Taenia laticollis*-specific DNA was recovered from 67% (8/12) of bilberry samples but not reliably from any of the lingonberry samples 0% (0/24), although the exposure dose was higher for those. The qPCR results suggest that under natural conditions, taeniid egg dispersion from the contamination spot is demonstrated but attachment is berry specific. The surface of bilberries may be more adhesive for taeniid eggs than the waxier and harder pericarp of the lingonberries or there might be a difference in the dispersal mechanism caused by different biotopes.

1. Introduction

Fresh forest berries are commonly known for their nutritional value and health benefits. *Vaccinium* berries have several positive health effects due to the special composition of the phytochemicals within them (Kowalska, 2021). Berries are often consumed raw. If not used for processed products, they are often not washed or heat-treated.

Taeniidae is a family of tapeworms that includes the following genera: *Echinococcus* spp., *Taenia* spp., *Hydatigera* spp. and *Versteria* spp. (Nakao et al., 2013). These genera include many parasites with significant veterinary and public health importance. Taeniid eggs are shown to be highly resistant to cold and even relatively high temperatures (Colli and Williams, 1972; Federer et al., 2015; Veit et al., 1995), so they are not easily destroyed in the environment. *Echinococcus* spp. infections are spread to humans via the faecal–oral

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route by the eggs of the parasite. Raw foodstuff is one transmission vehicle considered important. High public awareness is focused on the possible *Echinococcus multilocularis* infection risk associated especially with raw berries (e.g. Temesgen et al., 2021). Larval stages of the parasite can cause alveolar echinococcosis, a life-threatening disease for humans. Food-borne, or any route of transmission of *Echinococcus* spp. is difficult to prove because the incubation period is several years (Koutsoumanis et al., 2018), but epidemiological studies suggest that forest berries might be vehicles for *E. multilocularis* and cause consequent infections in humans (Kern et al., 2004). For Taeniidae, no standardized methods exist for detection of contamination in berries and raw vegetable foodstuffs (Chalmers et al., 2020). Methods have been developed and applied for the detection of other parasites, such as *Toxoplasma*, *Giardia*, *Cryptosporidium* (Chalmers et al., 2020) and *Cyclospora* (Murphy et al., 2018; Temesgen et al., 2021).

Recently, a lot of research has been directed at the risk of *E. multilocularis* contamination of foodstuffs, mainly fruits, berries and vegetables (Alvarez Rojas et al., 2018; Federer et al., 2016; Lass et al., 2015). In a recent meta-analysis, it was concluded that the possibility of alveolar echinococcosis transmission through ingestion of food and water contaminated with *E. multilocularis* eggs exists but that the potential food- and waterborne risk factors studied do not significantly increase the risk of infection (Conraths et al., 2017). Potential food- and waterborne risk factors were, for example, having a kitchen garden, going to the forest for vocational reasons and using a source of drinking water other than a well or tap. Plants growing near the ground and usually consumed raw, such as berries and lettuce, are likely vehicles. In forests or unprotected growing fields, definitive hosts can cause faecal contamination, but parasitic contamination can also occur through watering, soil or poor hygiene practices (Tefera et al., 2018). The eggs may be carried from the faeces by insects, water splash or wind. It was speculated that insects could disperse parasite eggs when *Taenia hydatigena* cysticerci were found in sheep on a remote island with no carnivore definitive hosts present (Torgerson et al., 1995). Red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*) and domestic dogs (*Canis lupus familiaris*) can excrete thousands to tens of thousands of *Echinococcus* eggs in their faeces during one day. The calculated mean biotic potential was approximately 300,000 eggs per infected animal in these carnivores (Kapel et al., 2006).

In our earlier study, we investigated whether taeniid eggs (*Taenia laticollis* as a model) can attach to the berry epicarp (surface) in natural conditions and developed a *T. laticollis*-specific semi-quantitative real-time polymerase chain reaction (qPCR) method for berry samples targeting the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene (Malkamäki et al., 2019). All the forest berry batches picked from the research plots contaminated by spraying of *T. laticollis* eggs 24 h earlier were found positive in qPCR; additionally, eggs were visualized in 20 of 21 bilberry and lingonberry samples. This indicates that taeniid eggs have the potential to attach to the berry surface, at least in the short term, through direct contamination. In the present study, we tested how efficiently taeniid eggs are dispersed from the contaminated fox faeces to the forest berries by mimicking the natural conditions. The collected berries were analysed by amplifying taeniid DNA with semi-quantitative qPCR. Non-zoonotic *T. laticollis* eggs were again used as a model since the parasite occurs naturally in Finland.

2. Materials and methods

2.1. Parasite eggs for inoculation

We used adult *T. laticollis* parasites to harvest eggs for the study. Intestinal *T. laticollis* worms were obtained from naturally infected lynx (*Lynx lynx*) carcasses submitted for pathological examination at the Finnish Food Authority in 2018–2019 and identified morphologically. Eggs were extracted by mincing the adult parasites in water suspension followed by isolation of the eggs by sieving (2 mm, 60 μm and 20 μm) and sedimentation. The eggs were then stored at −20 °C. An adult parasite was used as a positive DNA control. Red fox intestinal contents were obtained from animals submitted for combined surveillance of rabies, *Trichinella* and *E. multilocularis* at the Finnish Food Authority. The intestines were frozen at −80 °C for a week before analysis of the rectal contents for *E. multilocularis* according to the qPCR method (Isaksson et al., 2014), and the surplus was stored at −20 °C until thawed. Several thawed rectal contents were combined in a household kitchen blender (OBH Nordica Blendforce) with *T. laticollis* eggs to form 30 g piles of fox faeces artificially spiked with 30×10^3 eggs for bilberries and (later) 100×10^3 eggs for lingonberries. The numbers were different because of the presumption that lingonberries are less adhesive. The spiked faeces were packed in plastic vacuum bags using a household food vacuum sealer (OBH Nordica 7949 Supreme) and stored frozen at −18 °C until thawing and deposition.

2.2. Experimental contamination of the berry ground

Natural conditions with Myrtillus type (MT) and *Vaccinium* type (VT) (Cajander, 1926) harvested boreal forest with Scots pine (*Pinus sylvestris*) as the main tree species were surveyed for good bilberry (*Vaccinium myrtillus)* development in early July 2019 and for lingonberry (*Vaccinium vitis-idaea*) development in early August 2019 with the landowner's permission. The number of plots was four for bilberries in a single geographical location in MT forest (with some Oxalis-Majanthemum type, OMaT) (62° 14′ 46″ N 23° 08′ 18″ E, Kihniö, Finland) and eight for lingonberries in VT forest in one geographical location a couple of hundred metres from the bilberry plots. According to Cajander's (1926) classification, VT forest belongs to the dry moss (and lichen) forest class, MT to the moist moss forest class, and OMaT to the grass-herb forest class, VT being the most barren and OMT the most fertile or productive.

The experiment sites were established where numerous berry shrubs were blooming and marked with warning signs in Finnish, Swedish, English and Thai (due to the abundance of professional Thai wild berry pickers in Finland). The inoculated faecal piles were deposited in bilberry terrain on 4 July on stones and tree stumps to mimic the territorial faecal marking behaviour of red foxes. The lingonberry terrain faecal piles were similarly deposited on 7 August. After 41–42 days, the berries from the plots were picked utilizing commercial plastic berry picking tools, first picking the berries from the longest distance from the spiked faeces. The picking was

realized within a certain radius corresponding to concentric circles, the longest distance being 10–12 m for bilberry and 8–15 m for lingonberry, the middle distance 3 m and 7 m, and the closest distance 0 m and 2 m, respectively. The picking area of the lingonberries was larger than in the bilberry plots, because bilberry density appeared greater than lingonberry density. The berry batches were packed in individual plastic bags and frozen at −20 °C until analysis.

2.3. Washing of the eggs from the berry samples

The washing of the berries, DNA extraction and qPCR were performed using a method described in a previous study (Malkamäki et al., 2019). Each berry batch was washed separately in tap water containing 0.04% Tween 20 and shaken on an automatic rocker for 30 min at 50 rpm at room temperature. After this, the water was strained through 1 mm, 63 μm and 20 μm mesh sieves and the sediment centrifuged. The pellet formed was used for analysis immediately or stored at 4 °C for a maximum of 24 h.

2.4. DNA extraction

The total DNA was extracted from the sieving sediment with the Tissue and Hair Extraction Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions with some modifications to adapt the protocol for the sieving sediment. The incubation time was 2 h at 55 °C with 200 μl of incubation buffer containing proteinase K and dithiothreitol to shatter the eggshell. After this, the sample was vortexed for 5 s and centrifuged for 10 s at 2000 ×*g*. Centrifugation was added to the procedure to prevent the largest remaining berry particles from being carried to the DNA extraction tube. The supernatant was then transferred to a DNA extraction tube and 150 μl of lysis buffer and 14 μl of resin were added. After this, the protocol was continued according to the manufacturer's instructions. After extraction, the DNA was column purified with OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA,

Table 1
Semi-quantitative real-time polymerase chain reaction (qPCR) results for berry samples from plots experimentally contaminated with fox faeces inoculated with *Taenia laticollis* eggs.

Berry species	Number of eggs in faeces (× 10^3)	Identi- fication of the plot	Distance (m)	Mass (g)	Result (+/−)	qPCR mean Cq value (SD)	Melting temperature (°C)
Bilberry	30	B1	0–2	108	+	34.85 (0.92)	78 (±0.0)
	30	B1	3–7	251	+	34.37 (0.67)	78 (±0.0)
	30	B1	10–12	292	−	37.57 (1.47)	78 (±0.5)
	30	B2	0–2	339	+	34.23 (1.39)	78 (±0.5)
	30	B2	3–7	239	−	37.85 (2.37)**	78.5 (±0.0)
	30	B2	10–12	286	−	38.58 (1.31)	78.5 (±0.0)
	30	B3	0–2	207	+	35.45 (0.84)	78 (±0.0)
	30	B3	3–7	354	+	35.52 (1.18)	78 (±0.0)
	30	B3	10–12	288	−	37.14 (1.25)	78 (±0.0)
	30	B4	0–2	317	+	32.05 (1.88)	78 (±0.0)
	30	B4	3–7	379	+	33.23 (0.82)	78 (±0.0)
	30	B4	10–12	446	+	35.53 (1.17)	78 (±0.0)
Lingon- berry	100	L1	0–2	439	−	37.70 (0.81)	None
	100	L1	3–7	430	−	38.23 (0.66)	None
	100	L1	8–15	422	−	37.84 (0.68)	None
	100	L2	0–2	469	−	38.02 (1.33)	None
	100	L2	3–7	517	−	37.78 (1.39)	None
	100	L2	8–15	457	−	36.92 (0.59)	None
	100	L3	0–2	564	−	38.09 (0.57)	78.5 (±0.0)
	100	L3	3–7	499	−	0.00	None
	100	L3	8–15	295	−	36.79 (1.23)	78 (±0.0)
	100	L4	0–2	516	−	37.00 (2.06)**	None
	100	L4	3–7	550	−	37.11 (0.54)*	None
	100	L4	8–15	481	−	37.08 (0.99)	None
	100	L5	0–2	378	−	36.96 (0.99)	None
	100	L5	3–7	450	−	39.29 (0.00)***	None
	100	L5	8–15	388	−	37.25 (1.38)	78 (±0.0)
	100	L6	0–2	444	−	36.73 (0.79)	None
	100	L6	3–7	364	−	39.68 (0.00)***	None
	100	L6	8–15	334	−	37.03 (1.21)	83.5 (±0.0)
	100	L7	0–2	267	−	38.68 (0.13)**	None
	100	L7	3–7	351	−	37.78 (1.12)	83.5 (±0.0)
	100	L7	8–15	279	−	39.64 (0.44)**	None
	100	L8	0–2	323	−	38.24 (1.16)**	None
	100	L8	3–7	356	−	0.00	None
	100	L8	8–15	251	−	0.00	None

qPCR showed no quantification cycle (Cq) value once (*), twice (**) or three times (***) for qPCR performed in four replicates. SD = standard deviation. The readout of the reaction with melting temperatures of 77 ± 0.5 °C and a Cq value below the Cq of the detection limit (36.14) was used to confirm a positive reaction.

USA) following the manufacturer's instructions. The DNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the DNA was stored at −20 °C until qPCR.

2.5. qPCR assay

For the molecular detection of *T. laticollis*, DNA was amplified by using the partial mitochondrial *nad1* gene. The sequence of the forward primer is 5′-TCACAGTTTCGTAAGGGTCCAAAT-3′ (position 100–123) and the reverse sequence is 5′-CCAACTAACAACAACACCCCAGT-3′ (position 248–226), with a product size of 149 bp, as we have described previously (Malkamäki et al., 2019).

A qPCR assay was performed in white 96-well plastic plates in a final reaction volume of 20 µl consisting of 10 µl of 2 × SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.1 µl of 100 µM forward and reverse primer (Metabion, Martinsried, Germany), 2 µl of template DNA and 7.8 µl of nuclease-free water. Cycling conditions were as follows: an initial activation step of 98 °C for 3 min, followed by 40 cycles of denaturation at 98 °C for 15 s and annealing and elongation at 58 °C for 30 s using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The melt curve was constructed from 65 °C to 95 °C in 0.5 °C increments with a dwell time of 5 s at each temperature.

The read-out of the reaction with melting temperatures of 77 ± 1.0 °C and a quantification cycle (Cq) value below the Cq of the previously determined detection limit (36.14) was used to confirm a positive reaction. Positive amplification products were also confirmed by running them in 2% agarose gel stained with ethidium bromide. Each sample was amplified in duplicate in two separately performed qPCR runs (altogether four replicates) and the average Cq value and standard deviation were calculated. The amplification was analysed by using Bio-Rad CFX Maestro software. Two commercial bilberry batches bought from the market were used as negative control samples, and these were both negative in qPCR.

3. Results

The mass of the berries picked from the plots was greater for the lingonberries, ranging from 251 g to 564 g (average 409 g). For the bilberry samples, the mass ranged from 108 g to 446 g (average 292 g) (Table 1). In the bilberry samples showing positive amplification (Cq < 36.14 and Tm 77 ± 1 °C), Cq values were between 32.05 and 35.53 (average 34.40). Overall, 8 out of 12 (67%) of the bilberry samples tested positive for *T. laticollis* DNA in quantitative qPCR based on the mitochondrial *nad1* gene (Table 1). The lowest Cq values occurred in the amplification of the bilberry samples from the closest distance from the faecal piles, but the difference was only slight between the nearest and middle distances. Only in the nearest picking section (0–2 m) did all the bilberry plots show positive amplification. No lingonberry sample was positive for *T. laticollis* DNA (0/24; 0%). The Cq values were between 36.73 and no amplification at all in lingonberries from the plots with 100×10^3 eggs (Table 1). In the agarose gel, one distinct band was visible with the expected molecular weight (149 bp) in all the positive samples; no other bands were detected.

4. Discussion

The Taeniidae family contains many species with zoonotic potential. Contamination of the soil, water or fresh food products with eggs could be an important method of zoonotic transmission for many of these species, such as *Echinococcus* spp. We used non-zoonotic *T. laticollis* as a model for taeniids. The eggs of *Taenia* and *Echinococcus* spp. are similar in shape and size and their surface features are probably comparable (Lahmar et al., 2007; Smyth and Clegg, 1959); thus eggs of *T. laticollis* are representative of the family. Moreover, *T. laticollis* occurs naturally in lynxes and has no known zoonotic or pathogenic potential (Lavikainen et al., 2013); it therefore does not cause any specific risk to experimentally distribute it in nature. *Taenia* spp. helminths were found in 68% of the lynx intestine samples (Lavikainen et al., 2013), suggesting that they are common parasites among lynxes but most probably also among their prey, intermediate host lagomorphs. Due no lynxes or lynx tracks or faeces have been seen within the experimental plots, background contamination with *T. laticollis* eggs is not likely to have interfered the results.

Results in this study suggest that the dispersion and attachment of taeniid eggs is more efficient on to bilberries than on to lingonberries. They give evidence that eggs can disperse locally and the DNA can be detected after 40 days of experimental contamination. It is notable that here we investigated dispersal by *T. laticollis* DNA detection, not the presence or viability of the eggs. In former studies we showed that the DNA, as well as the eggs, can be found on the surface of both berries after 24 h following direct contamination with eggs (Malkamäki et al., 2019). This and the previous study also show that *T. laticollis* eggs still conserve their adhesive properties despite freezing and storing. Now the contamination was indirect (faecal pile in the area) and the detection time over 40 days, mimicking natural conditions.

It is interesting to note that the lingonberries had no specific amplification over the Cq value previously determined to be the limit of reliable detection despite the higher contamination dose. The number of eggs in this study was 30,000 for a faecal pile in the bilberry plots and 100,000 in the lingonberry plots. The number of eggs used was greater in the lingonberry plots, because it was assumed that the epicarp of lingonberries is less adhesive because of the smoother microstructure and greater amount of cuticular wax (Trivedi et al., 2019). It was decided that the picking area of the lingonberries was to be larger than that of the bilberry plots, because bilberry density was assumed greater than lingonberry density. However, after the 2019 berry season, it was noted that the bilberry yield was below average and the lingonberry yield was somewhat in the normal level. The yielded mass was after the adjustment higher for lingonberry samples than for bilberry samples, yet all the DNA results for lingonberries were negative. A larger quantity of berries lowers the detection limit with the same number of eggs due larger sediment. However, a larger number of berries (larger berry surface area for the eggs to attach) increases the potential of the sample to be positive. In our previous study (Malkamäki et al., 2019) using the same

protocol, the sensitivity of the overall method was 100% with five *T. laticollis* eggs in 100 g of bilberries or lingonberries, respectively, with mean Cq values varying between 30.90 and 32.82 with five eggs in bilberries and between 30.20 and 34.24 in lingonberries. This indicates that the protocol is working somewhat similarly with bilberries and lingonberries.

The surface composition and ultrastructure vary greatly between different berries (Trivedi et al., 2019). The epicarp of some berries, such as raspberries and strawberries, has a hair-like coating and it has been suggested that entrapment in surface structures in raspberries could be an attachment mechanism for *Cyclospora cayetanensis* oocysts (Sterling and Ortega, 1999). A study on the viability of *Toxoplasma gondii* oocysts after storage in refrigerator conditions compared the surface structures of bilberries and raspberries using scanning electron microscopy (Kniel et al., 2002). In the study, bilberry surface structures were observed, such as open stomata and crevices, to which oocysts considerably smaller than *Taenia* eggs could adhere. Stomata are pore-like structures in a plant epidermis that provide, for example, exchange of gases between the plant cells and surrounding air. Both berries used in the present study – bilberry and lingonberry – are relatively smooth and have a waterproof layer of a waxy substance called cutin. The amount of wax was 108.5 μg/cm^2 for bilberries and 871.1 μg/cm^2 for lingonberries in a study comparing wax amount and composition (Trivedi et al., 2019). This is one possible explanation for the taeniid eggs not being able to attach as easily or persistently to lingonberries compared with bilberries. Chemical composition of the wax affects epicuticular morphology in bilberry and wild type bilberries have dense rod-like formations and platelets in all of the latest developmental stages (Trivedi et al., 2021). To the authors' knowledge there are no studies describing whether parasitic eggs or other pathogens could be embedded in the waxy layer during ripening, but if eggs could be buried in the waxy layer during ripening, they would not be washed off from the berry surface easily. Because the cuticle plays an important role in preventing pathogen attack in plant organs, it would seem unlikely that particles would be trapped inside the wax, but it could hinder the attachment instead. This assumption is in line with our results.

Myrtillus type forest differs from VT forest regarding biodiversity and the abundance of invertebrates (Lakka and Kouki, 2009). Taeniid eggs can be transmitted mechanically by coprophagous arthropods that feed on faecal material, which can further contaminate berries, but the importance of this transmission route is not known (Jansen et al., 2021). The difference in positive samples between the berry species may have been due to the availability of different arthropods to act as vehicles of transmission in different forest types and at the different time of the summer. Experiments with *Taenia hydatigena* showed that infection can be transmitted to sheep on a pasture via blowflies that contain taeniid eggs (Lawson and Gemmell, 1990). Another experiment showed that blowflies can transport viable eggs from dog faeces to pigs fed with cooked meat (Lawson and Gemmell, 1990).

In Finland, bilberries typically ripen from late July until the beginning of September and lingonberries from the middle of August until the first snow in October, depending on the geographical location and weather conditions. Early summer weather conditions can also affect the ripening time. At the nearest weather station (Karvia Alkkia, Finnish Meteorological Institute) the mean temperature was 15.3 °C in July and 14.4 °C in August 2019. Eggs can disperse from the faecal pile with rain water splash (Alvarez Rojas et al., 2018). The difference between rainfall during the experiments was substantial; in July 2019 the rainfall was 28.9 mm and in August 2019 60.7 mm. It is thus possible that this difference in rainfall affected the adhesion of eggs to the surface of the berries. It is also notable that these conditions also represent the natural difference between the ripening periods of bilberries and lingonberries. A systematic review of the survival and dispersal of *Taenia* spp. eggs indicates that eggs survive best at a moderate temperature of 0–20 °C, but an even more important factor seems to be the humidity, with low humidity hindering survival (Jansen et al., 2021). Taeniid eggs can survive and remain infective in the field environment for a year (Jansen et al., 2021) and they are resistant to cold temperatures (Colli and Williams, 1972; Federer et al., 2016; Veit et al., 1995). In this study, the presence or viability of the eggs was not evaluated, but DNA detection gives an indirect indication of the contamination risk of, especially, bilberries even metres away from the faeces.

5. Conclusion

Taenia laticollis DNA was detected in all the bilberry samples from the nearest picking areas (0–2 m from the experimentally contaminated faecal pile) when picked after 41 days of placing the faeces and in some of the samples further away (up to 12 m). None of the lingonberry samples collected from similarly contaminated, but with an even higher amount of taeniid eggs, showed positive amplification in semi-quantitative qPCR. This indicates that bilberries might have more potential to serve as a transmission vehicle for taeniid eggs in natural conditions, most probably also including *E. multilocularis* and other zoonotic taeniids. This, however, should be confirmed with egg detection and identification of the dispersal mechanism.

Ethical standards

The study was performed in compliance with current Finnish national laws and regulations.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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