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A method for collecting eggs of *Pseudocapillaria tomentosa* (Nematoda: Capillariidae) from zebrafish *Danio rerio* and efficacy of heat and chlorine for killing the nematode's eggs

ML Martins^{1,2}, V Watral², JP Rodrigues-Soares¹, and ML Kent²

¹AQUOS - Aquatic Organism Health Laboratory, Aquaculture Department, Federal University of Santa Catarina (UFSC), Rod. Admar Gonzaga 1346, 88040-900, Florianopolis, SC, Brazil

²Departments of Microbiology and Biomedical Sciences, Oregon State University, 97331, Corvallis, OR, USA

Abstract

Pseudocapillaria tomentosa is a common pathogen of zebrafish (*Danio rerio*) in research facilities. We developed a method to collect and concentrate the nematode eggs using a modified sugar centrifugation method, and documented their normal development. Embryonating stages with blastomere formation followed by elongation of the embryo prior to larva formation cumulated in developed larvae inside the eggs and hatching after 5–10 d. We then evaluated the efficacy of heat and chlorine to kill them based on a larva development assay. Eggs were exposed to 40, 50, 60°C for 30 min and 1 h. Chlorine treatment was performed at 100, 250, 500, 1000, 3000 and 6000 ppm for 10 min. Samples exposed to 40°C for 30 min or 1 h showed incidences of larvated eggs similar to controls. In contrast, no larvation occurred with eggs exposed to either 50 or 60°C for 30 min or 1 h. Remarkably, in repeated assays, samples exposed to low doses of chlorine (100, 250, 500 and 1000 ppm for 10 min) showed significantly higher incidence of larvation than controls. Eggs treated with 3,000 ppm for 10 min did not develop larvae and no eggs were found after 6,000 ppm treatment.

Keywords

Zebrafish; Nematode; Egg collection technique; Treatment; Egg development

Introduction

The zebrafish, *Danio rerio* (Ham.) has become a very important laboratory model (Phillips & Westerfield 2014). In addition to developmental genetics, it is being used as a model to study the chronic granulomatous infectious diseases caused by *Mycobacterium* spp. (Chang *et al.* 2015), microsporidia (Troemel 2011; Sanders, Peterson & Kent 2014), *Toxoplasma gondii* (Sanders *et al.* 2015) and nematodes (Collymore *et al.* 2014). Zebrafish has been used in behavior studies after exposure to drugs (Richendrfer *et al.* 2012), toxicity assays (Mu *et*

al. 2016) and also in studies involving stress and psychiatry due to its similarity with the organization and stress-regulating systems in humans and rodents (Steenbergen *et al.* 2011). With the increase use of adult zebrafish as research endpoints there is concurrently more concern about the impacts of underlying parasite infections in the fish (Kent, Harper & Wolf 2012). Capillarid and camallanid nematodes are one of the most important helminth parasites in ornamental fishes, such as the guppy *Poecilia reticulata* Peters (Evans & Lester 2001), the platy *Xiphophorus maculatus* Günther (Piazza *et al.* 2006; Martins *et al.* 2007) and the angel fish *Pterophyllum scalare* (Schultze) (Thilakaratne *et al.* 2003). Moravec, Gelnar & Rehulka (1987a) reported mass mortality in the ornamental reared armoured catfish *Ancistrus dolichopterus* Kner due to heavy infections by *Capillostrongyloides ancistri*. Abdominal swelling, protruded anus, anorexia as well as haemorrhages, edema, epithelial erosion and inflammatory infiltrate were reported in beta *Betta splendens* Regan and guppies parasitized by *Camallanus cotti* (Menezes *et al.* 2006)

We are studying *Pseudocapillaria tomentosa* (Dujardin 1843) Moravec 1987 as it has been recognized as an important parasite of zebrafish research facilities (Kent *et al.* 2002; 2012; Murray & Peterson 2015). The nematode belongs to trichinelloid nematodes and the genus is recognized to infect in cyprinid and other fish families (Moravec 2001), particularly ornamental fishes (Moravec, Ergens & Repová 1984). *Pseudocapillaria brevispicula* (Linstow 1873) is considered a junior synonym of *P. tomentosa* (Moravec, Prokopic & Shlikas 1987b; Moravec, Wolter & Körting 1999). The nematode is the second most common parasitic infection in zebrafish held in research facilities, and based on diagnostic submissions to the Zebrafish International Resource Center (ZIRC), the infection occurred in up to 20% of zebrafish laboratories between 2006–2014 (Kent *et al.* 2016). As observed in *Pseudorashbora parva* Temminck & Schlegel (Mihok *et al.* 2011), it causes chronic emaciation and chronic mortalities in zebrafish (Kent *et al.* 2002). Histological examination of infected fish reveals, profound chronic enteritis and occasionally coelomitis (Kent *et al.* 2002; Murray & Peterson 2015), and Balla *et al.* (2010) showed that infected fish had significantly increased eosinophils. Spitsbergen *et al.* (2000) found that infected zebrafish that were exposed to a low dose of the carcinogen dimethylbenze[a]anthracene had a higher incidence of gastrointestinal tumors than similarly exposed fish that were not infected. Therefore, subclinical infections are also of concern in zebrafish as they may cause non-protocol induced variation in research (Kent *et al.* 2012).

As with other capillariids, this nematode is oviparous and their unembryonated eggs are found in fish feces. Moravec (1983) obtained eggs of *P. tomentosa* (which was called *P. brevispicula* at that time) from gravid females collected from the intestines of tench *Tinca tinca* (L.) and the worms larvated and became infectious after 7 days at 20–22°C. *Pseudocapillaria tomentosa* has been reported to use paratenic hosts such as oligochaete worms, but the parasite is directly transmissible in zebrafish research colonies (Kent *et al.* 2002).

As with many other pathogens in aquaculture, control of *P. tomentosa* infections in zebrafish can be achieved by avoidance and quarantine, treatment of infected fish, and disinfection of water exposed to the parasite. Three recent studies reported that zebrafish infected with *P. tomentosa* can be efficaciously treated with classes of antiparasitic compounds that are

commonly used against nematodes, such as macrocyclic lactones and benzimidazoles (Maley *et al.* 2013; Collymore *et al.* 2014; Samaee 2015).

Most zebrafish in research are reared in large, multi-aquaria recirculating systems, and hence it would also be important to develop protocols to destroy the parasite's eggs within these systems. Nematode eggs are notoriously resistant to disinfection (Bratanov 1969; de Souza *et al.* 2011). Here we evaluated the efficacy of chlorine and heat to kill the eggs. We selected these agents as chlorine is used both for decontaminating zebrafish eggs and as a general disinfectant in zebrafish and other aquaculture facilities (Westerfield 2007; Harper & Lawrence 2011). Regarding heat, whereas the eggs of many terrestrial nematodes are tolerant to a wide range in temperature, we hypothesized that those of aquatic nematodes may be less tolerant to very high temperatures as they have not evolved in such environments. Moreover, disinfection of holding systems with heat would provide a method that would not involve the use of potentially corrosive as well as toxic chemicals.

To conduct these evaluations we needed to develop a protocol for collecting and concentrating reasonable numbers of viable eggs. There are numerous protocols for collecting eggs from feces for intestinal nematodes of terrestrial animals, some of which involve separation and concentration with density dependent fecal flotation by centrifugation (Ballweber *et al.* 2014). With helminths of fish, Marchiori *et al.* (2013) developed a method to concentrate monogenean eggs shed from fish, which consisted of a 20 L plastic bottle containing the hosts where the eggs were collected in a central chamber with 41 μm mesh size. The aim of this study was to standardize a method for collection of *P. tomentosa* eggs from zebrafish, describe the development of the parasite up to the time of hatching, and to evaluate alternatives in controlling the egg development by exposing them to heat and chlorine.

Material and Methods

Fish and parasite maintenance

Nematode eggs for this study were obtained from 60 previously exposed zebrafish kept in a 70 L aquarium, with constant water flow and aeration, fed approximately 2% of body weight per day with powdered mixed diet used previously at the SARL Lab (Miller *et al.* 2014). Fish were infected by co-habitation with parasitized fish. The water quality was monitored and remained as follows: temperature $27.7\pm0.7^{\circ}\text{C}$, dissolved oxygen $6.6\pm0.2\text{ mg.L}^{-1}$, pH 7.6 ± 0.1 and ammonia $0.2\pm0.05\text{ mg.L}^{-1}$. The fish were wildtype 5D from the Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University, Corvallis, OR, USA

Nematode egg collection

Infected fish were distributed into four small, static polyurethane tanks (1.6 L) capacity in which fish were placed in a Thoren breeding cage (Thoren Aquatic Systems, Hazelton, PA) with 15 fish per tank with aeration. The fish were held in the breeding device with a screen bottom suspended above the bottom of the tank (approximately 10 mm). This allowed the nematode eggs to fall through to the bottom of the tank and keep them isolated from fish.

During this period the water quality was kept at: dissolved oxygen 6.5–7.0 mg.L⁻¹, pH 7.6–7.8, ammonia 0.15–0.25 mg.L⁻¹, and fish were not fed during the procedure. After three days, the fish were removed and the water left to settle for 2 h. About three quarters of the water was carefully siphoned from each tank near the surface to avoid eggs that accumulated on the bottom. The remaining contents of the tanks were then placed in six 250 mL centrifuge bottles and centrifuged at 1,468 g for 30 min (Sorvall® RC-5B Du Pont Instruments, Connecticut, USA). The supernatant was carefully removed, the centrifuged content placed in 14 mL plastic conical tubes and centrifuged at 262 g for 5 min using a Unico Powerspin™ MX centrifuge (New Jersey, USA). The supernatant was removed and 1 mL of saturated sugar solution (454 g sucrose in 355 L hot tap water, allowed to cool to room temperature) was added, mixed with a vortex mixer. This resulted in a specific gravity of 1.27. The tubes were then filled to almost the top with the sugar solution, and then centrifuged again at 262 g for 5 min. The tubes were then filled completely to the top with the sugar solution until the formation of a bulging meniscus was achieved. A glass coverslip was then placed on the top of the meniscus and left to rest for 75 min. The coverslips were then carefully removed and washed into a sterile Petri dish (35 mm diameter) with the aid of a micropipette containing about 1 mL sterile water from our fish system with the addition of Tween 20® at 0.05% to facilitate the sugar solution dilution and suspension of the eggs. This content with eggs were then placed in Falcon tubes (14 mL), centrifuged at 262 g for 5 min to obtain the desired volume (approximately 600 µL) of water for the assays. This water solution contained 1:100 penicillin streptomycin (Penstrep, Invitrogen® New York, USA). After the appropriate heat or chlorine treatments, 50 µL of water containing the eggs were added to depression slides with a coverslip overlaid. The slides were placed in Petri dishes (100 mm diameter) with wet paper towels under the slides to maintain humidity, and incubated at 28°C. Slides were examined daily at 100 or 400 x magnification for 10 days. Eggs were scored as either 1) embryonating (intact but no larvae), 2) larvated, or 3) dead (Fig. 1, 2). Live and embryonating eggs were characterized similar to that previously reported for *Trichuris suis* (Beer 1973; Vejzagic *et al.* 2016) and *Trichuris* spp. (Steinmann *et al.* 2015) - i.e., unsegmented embryo, formation of blastomeres, small blastula formed by blastomeres, or cylindrical embryo (characterized by prolongation at one side of embryo that consists in larva formation). Eggs were scored as dead when the content inside the eggs was clear and not dense, or they lost the plugs at the end of the eggs. At later time points it was important to distinguish between the latter conditions versus eggs that were empty simply due to hatching. Eggs were scored as larvated when developed, elongated larvae were visible, and these larvae often motile within the egg. Photomicrographs were made with a Leica microscope (DMLB 100S, Germany) using a SPOT photomicrography system (Diagnostics Instruments Inc, Sterling Heights, MI).

Heat treatment

Heat was applied by placing the eggs in about 1 mL in 1.5 mL Eppendorf tubes in a water bath. Five separate assays consisted of four treatments in triplicate as follows (Table 1): Assay 1 and 2, 40, 50 and 60°C for 30 min; Assay 3, 4 and 5, 28, 40, 50 and 60°C heating exposure for 1 h. The tubes were then centrifuged at 262 g for 5 min to concentrate the eggs again, and they were then divided into 4 depression slides, a coverslip was overlaid and examined over for 10 d as described above.

Chlorine treatment

Four separate assays consisted of three treatments in quadruplicates after chlorine treatment. Firstly, eggs were exposed to the appropriate concentrations of chlorine made using a 5% stock solution J.T. Baker (Avantor Performance Materials, Center Valley, PA) mixed with 100 mL water from our fish facility. The pH was 7-2-7.6 for each solution. For exposures, the egg mixture in Falcon tubes was reduced to a pellet with a minimum amount of water. After 10 min treatment with chlorine solution the contents in was neutralized with sodium thiosulphate at equal volume to the exposure mixture at twice the chlorine concentration. Control tubes also received sodium thiosulphate. The tubes were then centrifuged at 262 g for 5 min to concentrate the eggs again, fish system water was added, and they were then divided into 4 depression slides with a coverslip was overlaid. Slides were then examined for 10 d as described above. Assay 6 and 8 consisted of 0 (controls), 500 and 1,000 ppm chlorine; Assay 7 was control, 3,000 and 6,000 chlorine, and Assay 9 was control, 100 and 250 ppm chlorine.

Statistical analysis

Kolmogorov-Smirnov and Bartlett tests were used to verify data normality and homoscedasticity. Mauchly's test and the lower bound, Huynh & Feldt and Geisser-Greenhouse corrections were performed to verify sphericity. The data were submitted to repeated measures analysis of variance (ANOVA). Tukey test was used for mean comparison. All analysis were performed with STATISTICA (Statsoft) software. When needed, data were transformed and the significance level was $\alpha = 0.05$.

Results

Nematode egg collection and development

The number of eggs used in each assay was as follows: Assay 1 (83 eggs); Assay 2 (246 eggs); Assay 3 (82 eggs); Assay 4 (159 eggs); Assay 5 (368 eggs); Assay 6 (386 eggs); Assay 7 (420 eggs); Assay 8 (352 eggs); Assay 9 (416 eggs). While almost all eggs were scored as live/unembryonated or dead at time zero, occasionally a few were embryonated or larvated at this time, presumably representing developing eggs that the fish had previously ingested rather than newly released eggs from worms within the fish. Egg development began with small blastomeres (Fig. 1 A), large blastomeres (Fig. 1 B), followed by initial differentiation into elongation of the embryo (Fig. 1 C), further elongation of the embryo, including development of flexure occurred at 5 days (Fig. 1 D). Formation of distinct larval worms (Fig. 1 E) and hatched larva (Fig. 2 A–B) occurred between 5 and 10 days after incubation, but occasionally earlier at 3 days. Dead eggs present clear content, the plugs are not well defined (Fig. 2 C–D).

The most pertinent and clear results pertained to comparisons of incidence and total percent of larvated eggs subjected to the various treatments, and these results are presented in Figs. 3–7. Data pertaining to dead and intact (but not larvated) eggs, with further statistical analyses are presented in Tables 1–4.

Heat treatment

Two separate assays showed that treatment of eggs for 50 or 60°C for 0.5 h was effective for killing eggs based on the absence of larvae (Fig. 3). In contrast, 40°C was not effective, and eggs held at this temperature exhibited similar patterns of larvation compared to the controls. Non-treated eggs showed no significant difference ($P>0.05$) when compared to those treated at 40°C both after 30 min and 1 h heating. In other words, eggs exposed to 40°C did show fewer larvated eggs than controls in Assay 2, but this was not statistically different. Increasing the duration of heat exposure to 1h (Figs. 4) did not reliably kill eggs at 40°C as about 40% of the eggs larvated at this temperature in Assay 3 (Fig. 4). No eggs larvated at 40°C in Assays 4 and 5 (Fig. 4), but these results are difficult to interpret as few control eggs larvated in these assays. Eggs were considered dead if they did not larvate after 10 d and consisted of only shells and no internal content. In other words, some eggs exposed to higher temperatures were not scored as “dead” (Table 1) or scored as “embryonated” (Table 2) at the end of the assays.

Chlorine treatment

Chlorine at lower concentrations of (100, 250, 500, 1000 ppm) was not effective at killing the nematode eggs (Figs. 5–6). Non-treated eggs and those treated with 100 or 250 ppm showed larval development at 3 d with a high incidence of larval development 5 and 7d. Increasing the chlorine concentration to 500 and 1000 ppm showed similar results, but at these concentrations survival was actually significantly higher ($P<0.05$) compared to that of non-treated eggs. Remarkably, in both of assays using 500 and 100 ppm there was poor survival of control eggs, while eggs exposed to chlorine at 500 ppm showed a high incidence of larvation in both assays, and in one assay eggs exposed to 1,000 ppm chlorine larvated at >50% incidence. In the second assay, lower ($P<0.05$) percentage of dead eggs was observed in 500 and 1000 ppm 10 days after treatment compared to non-treated eggs

Eggs treated with 3000 and 6000 ppm for 10 min did not developed larvae (Fig. 7). Concentrations of 3000 and 6000 ppm caused 76 % mortality in eggs one day after treatment and 100% mortality 10 days after treatment (Table 3). At 7 d, eggs treated with 3000 ppm even showed 7–9% undeveloped eggs (Table 4), but by 10 d all the eggs were scored as dead. At 6,000 ppm the eggs were completely destroyed and hence no eggs were observed.

Discussion

The sequence and time of development observed here were very consistent to that observed for *P. tomentosa* eggs held at 22°C reported by Moravec (1983). Our results were also similar to those found reported for *Capillaria columbae* from pigeons, in which eggs completed larvation in 8 d at 24°C (Wehr 1939). Capillarid nematodes are related to members of the genus *Trichuris* in that both are classified within family Trichuridae and have eggs with bipolar plugs. The development that we observed was also similar to that described for *T. suis* (Beer 1973; Vejzagic *et al.* 2016) in presenting different stages of development, firstly without egg division (unsegmented), small and large blastomeres, cylindrical formation of embryo until the larval development and hatching.

Eggs of terrestrial parasitic nematodes can develop at higher temperatures, and temperatures greater than 40°C are usually needed to kill them (Jimenez 2007). For example, *T. suis* developed rapidly at 30–34°C (Vejzagic *et al.* 2016) and Tharaldsen & Helle (1989) reported that up to 27% of *Ascaris* spp. eggs continued their development at 37°C. Barnard *et al.* (1987) found that 50% *Ascaris lumbricoides* eggs survived at 50°C and Shafir *et al.* (2007) reported more than 75% of the eggs of *Baylisascaris procyonis* remained viable at 52–57°C and were only inactivated at 62°C. Our observations of larvation of *P. tomentosa* eggs at 40°C was surprising because we assumed that through evolutionary time neither the worm or its eggs would ever be exposed to this temperature. It must be noted that we considered eggs “dead” when they failed to larvate after 10 d (Figs. 2–7). In other words, some eggs exposed to 50 or 60°C, or high chlorine concentrations as discussed below, were scored as embryonated, but not larvated, at 10 d. We conclude that they would not eventually larvate and be infectious at a later time point.

We found that chlorine at 3,000 ppm was effective at killing the eggs of *P. tomentosa*. In contrast, chlorine exposure at lower doses was not effective and we observed enhanced larval development at 500 and 1,000 ppm. Similar findings were found by Ogunfowora & Evans (1977), who reported that chlorine at 4,000 ppm stimulated the hatch of *Meloidogyne naasi* (Nematoda: Tylenchida) eggs parasite of barley root. Sodium hypochlorite (chlorine) at 0.4% (4,000 ppm) for 10 min was effective for killing members of other nematode genera found in soil (Esser 1972) and 1% (10,000 ppm) chlorine destroyed the cysts of the nematode *Globodera rostochinensis* (Tylenchida) from potatoes tubers (Wood & Foot 1977). On the other hand, 10 ppm chlorine did not completely kill the eggs of the rat pinworm nematode *Syphacia muris* (Miyaji, Kamiya & Shikata 1988).

Our observations of enhanced larvation at some of the lower concentrations of chlorine suggests that these conditions provided an unknown stimulation for egg development, consistent with some observations from terrestrial nematodes. For example, larvae of *Ascaris suum* hatched up to 90% by agitating them with glass beads incubated at 37°C for 30–45 min (Urban, Douvres & Tromba 1981). Among several stimulation methods used to promote the egg hatching of *T. suis*, mechanical stimulation with glass beads was the most also effective (Vejzagic *et al.*, 2015). Microwave irradiation has been reported to stimulate the growth of *Carnorhabditis elegans* L₁ larvae (Pomerai *et al.* 2002). Two alternatives remain unexplained and deserve future evaluation: the first is that possibly the chlorine treatment provoked a shock on the eggs similar to that found when eggs were stimulated with glass beads (Vejzagic *et al.* 2015) and the second one is that the low chlorine concentration could have improved the water quality in the microenvironment of the slides.

Certain recommendations can be put forth from our study regarding practical applications for control of the infection in zebrafish colonies. Regarding heat, as 50°C or higher kills the eggs, this approach could be used to disinfect a recirculating system after the fish are removed. Further experiments should be conducted to determine if perhaps lower heat (e.g., 40–45°C) could kill the eggs if applied for a longer duration, such as 12–24 h, which still would be a practical approach. Regarding chlorine, whereas the parasite does not infect ovaries or testis, there is a risk of maternal transmission during spawning. Zebrafish eggs are usually treated with chlorine at 25–50 ppm for about 10 min, but the concentration needed

for killing *P. tomentosa* eggs is much higher than the lethal concentrations for zebrafish embryos (Kent *et al.* 2014). Nevertheless, the effective levels for the nematode eggs at 3,000 ppm is reasonable for surface disinfection and water disinfection in structures not holding fish. Other disinfectants used to treat fish eggs or containers and surfaces without fish, such as iodine and hydrogen peroxide, should be investigated. Last, ultraviolet (UV) disinfection of water returning to tanks is routinely used in recirculating fish systems, and there are reports of the efficacy of UV against nematode eggs in aquaculture (Lakeh 2014). Hence this should be explored with the eggs of *P. tomentosa*.

Apart from the recommendations suggested by Kent *et al.* (2009), we also suggest that ornamental or zebrafish facilities conduct periodic analysis of the fish feces by using the method that we describe here. An entire population could be non-lethally examined with our method by collecting feces from specific tanks, or from fish held in individual tanks to determine a more precise prevalence of infection. This protocol could be adapted for other intestinal helminths and fish hosts in aquaculture, and may lead to alternatives to the traditional anthelmintic treatments, which could detrimental side effects (Taraschewski, Renner & Mehlhorn 1988).

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References

- Balla KM, Lugo-Villarino G, Spitsbergen JM, Stachura DL, Hu Y, Bañuelos K, Romo-Fewell O, Aroian RV, Traver D. Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants. *Blood*. 2010; 116:3944–3954. [PubMed: 20713961]
- Ballweber LR, Beugnet F, Marchiondo AA, Payne PA. American Association of Veterinary Parasitologists' review of veterinary fecal flotation methods and factors influencing their accuracy and use-Is there really one best technique? *Veterinary Parasitology*. 2014; 204:73–80. [PubMed: 24893692]
- Barnard RJ, Bier JW, Jackson GJ, McClure FD. *Ascaris lumbricoides suum*: thermal death time of unembryonated eggs. *Experimental Parasitology*. 1987; 64:120–122. [PubMed: 3609225]
- Beer RJS. Morphological descriptions of the egg and larval stages of *Trichuris suis* Schrank, 1788. *Parasitology*. 1973; 67:263–278. [PubMed: 4761767]
- Bratanov V. The ovocide action of certain disinfection agents on the eggs of *Trichocephalus ovis* and *Ascaris suum* in vitro. *Veterinary Science*. 1969; 6:79–86.
- Chang CT, Colicino EG, Di Paola EJ, Al-Hasnawi HJ, Whipps CM. Evaluating the effectiveness of common disinfectants at preventing the propagation of *Mycobacterium* spp. isolated from zebrafish. *Comparative Biochemistry and Physiology Part C*. 2015; 178:45–50. [PubMed: 26423444]
- Collymore C, Watral V, White JR, Colvin ME, Rasmussen S, Tolwani RJ, Kent ML. tolerance and efficacy of emamectin benzoate and ivermectin for the treatment of *Pseudocapillaria tomentosa* in laboratory zebrafish (*Danio rerio*). *Zebrafish*. 2014; 11:490–497. [PubMed: 25237985]
- de Souza GSMB, Rodrigues LA, Oliveira WJ, Chernicharo CAL, Guimarães MP, Massara CL, Grossi PA. Disinfection of domestic effluents by gamma radiation: effects on the inactivation of *Ascaris lumbricoides* eggs. *Water Research*. 2011; 45:5523–5528. [PubMed: 21911240]
- Esser RP. Effect of sodium hypochlorite concentrations on selected genera of nematodes. *Proceedings of the Helminthological Society of Washington*. 1972; 39:108–114.

- Evans BB, Lester RJ. Parasites of ornamental fish imported into Australia. *Bulletin of the European Association of Fish Pathologists*. 2001; 21:51–55.
- Harper, C.; Lawrence, C. *The Laboratory Zebrafish*. CRC Press; Boca Raton, FL: 2011.
- Jimenez B. Helminth ova removal from wastewater for agriculture and aquaculture reuse. *Water Science & Technology*. 2007; 55:485–493. [PubMed: 17305174]
- Kent ML, Harper C, Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. *ILAR Journal*. 2012; 53:126–134. [PubMed: 23382344]
- Kent ML, Bishop-Stewart JK, Matthews JL, Spitsbergen JM. *Pseudocapillaria tomentosa*, a nematode pathogen, and associated neoplasms of zebrafish (*Danio rerio*) kept in research colonies. *Comparative Medicine*. 2002; 52:362–366.
- Kent ML, Feist SW, Harper C, Hoogstraten-Miller S, Mac Law J, Sánchez-Morgado JM, Tanguay RL, Sanders GE, Spitsbergen JM, Whipps CM. Recommendations for control of pathogens and infectious diseases in fish research facilities. *Comparative Biochemistry and Physiology Part C*. 2009; 149:240–248. [PubMed: 18755294]
- Kent ML, Buchner C, Barton C, Tanguay RL. Toxicity of chlorine to zebrafish embryos. *Diseases of Aquatic Organisms*. 2014; 107:235–240. [PubMed: 24429474]
- Kent, ML.; Spitsbergen, JM.; Matthews, JM.; Fournie, JW.; Murray, KN.; Westerfield, M. *ZIRC Health Services Zebrafish Disease Manual*. Zebrafish International Resource Center, US Environmental Protection Agency; Gulfbreeze, Florida: 2016. Diseases of zebrafish in research facilities.
- Lakeh, AAB. Dissertation. Leibniz-Institute of Freshwater Ecology and Inland Fisheries; Berlin, Germany: 2014. Effect of low frequency ultrasound and ultraviolet-C light for water disinfection in recirculating aquaculture systems; p. 72
- Maley D, Laird AS, Rinkwitz S, Becker TS. A simple and efficient protocol for the treatment of zebrafish colonies infected with parasitic nematodes. *Zebrafish*. 2013; 10:447–450. [PubMed: 23808385]
- Marchiori N, Tancredo K, Roumbedakis K, Gonçalves ELT, Pereira J Jr, Martins ML. New technique for collecting eggs from monogenean parasites. *Experimental Parasitology*. 2013; 134:138–140. [PubMed: 23500621]
- Martins ML, Garcia F, Piazza RS, Ghiraldelli L. *Camallanus maculatus* n. sp. (Nematoda: Camallanidae) in an ornamental fish *Xiphophorus maculatus* (Osteichthyes: Poeciliidae) cultivated in São Paulo State, Brazil. *Brazilian Journal of Veterinary and Animal Science*. 2007; 59:1224–1230.
- Menezes RC, Tortelli R, Tortelli-Neto R, Noronha D, Pinto RM. *Camallanus cotti* Fujita, 1927 (Nematoda, Camallanoidea) in ornamental aquarium fishes: pathology and morphology. *Memórias do Instituto Oswaldo Cruz*. 2006; 101:683–687. [PubMed: 17072484]
- Mihok T, Kosuth P, Kocisová A, Perkárik L, Bártošová E, Major P. The intestinal parasite *Pseudocapillaria tomentosa* (Dujardin, 1843) of the invasive fish species topmouth gudgeon, *Pseudorasbora parva* (Temminck and Schlegel), in Slovakia. *Journal of Fish Diseases*. 2011; 34:711–714. [PubMed: 21777250]
- Miller GW, Truong L, Barton CL, Labut EM, Bebold KM, Traber MG, Tanguay RL. The influences of parental diet and vitamin E intake on the embryonic zebrafish transcriptome. *Comparative and Biochemical Physiology: Genomics and Proteomics*. 2014; 10:22–29.
- Miyaji S, Kamiya M, Shikata J. Ovicidal effects of heat and disinfectants on *Syphacia muris* estimated by in vitro hatching. *Jikken Dobutsu*. 1988; 37:399–404. [PubMed: 3240778]
- Moravec F. Observations on the bionomy of the nematode *Pseudocapillaria brevispicula* (Linstow, 1873). *Folia Parasitologica*. 1983; 30:229–241.
- Moravec, F. Trichinelloid nematodes parasitic in cold-blooded vertebrates. *Academia; Praha*: 2001. p. 464
- Moravec F, Ergens R, Repová R. First record of the nematode *Pseudocapillaria brevispicula* (Linstow, 1873) from aquarium fishes. *Folia Parasitologica*. 1984; 31:241–245.
- Moravec F, Gelnar M, Rehulka J. *Capillostrongyloides ancistri* sp. n. (Nematoda: Capillariidae) a new pathogenic parasite of aquarium fishes in Europe. *Folia Parasitologica*. 1987a; 34:157–161. [PubMed: 3596394]

- Moravec F, Prokopi J, Shlikas AV. The biology of nematodes of the family Capillaridae Neveu-Lemaire, 1936. *Folia Parasitologica*. 1987b; 34:39–56. [PubMed: 3583129]
- Moravec F, Wolter J, Körting W. Some nematodes and acanthocephalans from exotic ornamental freshwater fishes imported into Germany. *Folia Parasitologica*. 1999; 4:296–210.
- Mu X, Chai T, Wang K, Zhu L, Huang Y, Shen G, Li Y, Li X, Wang C. The developmental effect of difenoconazole on zebrafish embryos: A mechanism research. *Environmental Pollution*. 2016; 212:18–26. [PubMed: 26840512]
- Murray KN, Peterson TS. Pathology in practice. *Journal of the American Veterinary Medical Association*. 2015; 246:201–203. [PubMed: 25554935]
- Ogunfowora AO, Evans AAF. Factors affecting the hatch of eggs of *Meloidogyne naasi*, an example of diapause in a second stage larva. *Nematologica*. 1977; 23:137–146.
- Piazza RS, Martins ML, Guiraldelli L, Yamashita MM. Parasitic diseases of freshwater ornamental fishes commercialized in Florianópolis, Santa Catarina, Brazil. *Boletim do Instituto de Pesca*. 2006; 32:51–57.
- Phillips JB, Westerfield M. Zebrafish models in translational research: tipping the scales toward advancements in human health. *Disease Models & Mechanisms*. 2014; 7:739–743. [PubMed: 24973743]
- Pomerai DI, Dawe A, Djerbib L, Allan J, Brunt G, Daniells C. Growth and maturation of the nematode *Caenorhabditis elegans* following exposure to weak microwave fields. *Enzyme Microbial Technology*. 2002; 30:73–79.
- Richendrfer H, Pelkowski SD, Colwill RM, Créton R. Developmental sub-chronic exposure to chlorpyrifos reduces anxiety-related behavior in zebrafish larvae. *Neurotoxicology and Teratology*. 2012; 34:458–465. [PubMed: 22579535]
- Samaee SM. Experimental assessment of the efficacy of five veterinary broad-spectrum anthelmintics to control the intestinal capillariasis in zebrafish (*Danio rerio*). *Zebrafish*. 2015; 12:255–267. [PubMed: 25856204]
- Sanders JL, Peterson TS, Kent ML. Early development and tissue distribution of *Pseudoloma neurophilia* in the zebrafish, *Danio rerio*. *Eukaryotic Microbiology*. 2014; 61:238–246.
- Sanders JL, Zhou Y, Moulton HM, Moulton ZX, McLeod R, Dubey JP, Weiss LM, Kent ML. The zebrafish, *Danio rerio*, as a model for *Toxoplasma gondii*: an initial description of infection in fish. *Journal of Fish Diseases*. 2015; 38:675–679. [PubMed: 25951508]
- Shafir SC, Wang W, Sorvillo FJ, Wise ME, Moore L, Sorvillo T, Eberhard ML. Thermal death point of *Baylisascaris procyonis* eggs. *Emerging Infectious Diseases*. 2007; 13:172–173. [PubMed: 17370540]
- Spitsbergen JM, Tsai HW, Reddy A, Miller T, Arbogast D, Hendricks JD, Bailey GS. Neoplasia in zebrafish (*Danio rerio*) treated with 7,12-Dimethylbenze[a]anthracene by two exposure routes at different developmental stages. *Toxicologic Pathology*. 2000; 28:705–715. [PubMed: 11026607]
- Steenbergen PJ, Richardson MK, Champagne DL. The use of the zebrafish model in stress research. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*. 2011; 35:1432–1451. [PubMed: 20971150]
- Steinmann P, Rinaldi L, Cringoli G, Du ZW, Marti H, Jiang JJ, Zhou H, Zhou XN, Utzinger J. Morphological diversity of *Trichuris* spp. eggs observed during an anthelmintic drug trial in Yunnan, China, and relative performance of parasitological diagnostic tools. *Acta Tropica*. 2015; 141:184–189. [PubMed: 25174679]
- Taraschewski H, Renner C, Mehlhorn H. Treatment of fish parasites. 3. Effects of levamisole HCl, metrifonate, fenbendazole, mebendazole, and ivermectin on *Anguillicola crassus* (nematodes) pathogenic in the air bladder of eels. *Parasitology Research*. 1988; 74:281–289. [PubMed: 3362833]
- Tharaldsen J, Helle O. Survival of parasite eggs in livestock slurry for compost heat. *Acta Agriculturae Scandinavica*. 1989; 39:381–387.
- Thilakarathne ID, Rajapaksha G, Hewakopara A, Rajapakse RP, Faizal AC. Parasitic infections in freshwater ornamental fish in Sri Lanka. *Diseases of Aquatic Organisms*. 2003; 54:157–162. [PubMed: 12747641]

- Troemel ER. New models of microsporidiosis: infections in zebrafish, *C. elegans*, and honey bee. *Plos Pathogens*. 2011; 7:1–4.
- Urban JF Jr, Douvres FW, Tromba FG. A rapid method for hatching *Ascaris suum* eggs in vitro. *Proceedings of the Helminthological Society of Washington*. 1981; 48:241–243.
- Vejzagic N, Thamsborg SM, Kringel H, Roepstorff A, Bruun JM, Kapel CMO. In vitro hatching of *Trichuris suis* eggs. *Parasitology Research*. 2015; 114:2705–2714. [PubMed: 26008635]
- Vejzagic N, Kringel H, Bruun JM, Roepstorff A, Thamsborg AM, Grossi AB, Kapel CMO. Temperature dependent embryonic development of *Trichuris suis* eggs in a medicinal raw material. *Veterinary Parasitology*. 2016; 215:48–57. [PubMed: 26790737]
- Wehr EE. Studies on the pigeon capillariid, *Capillaria columbae*. US Department of Agriculture Technical Reports. 1939; 679:1–18.
- Westerfield, M. The Zebrafish Book, 5th Edition; A guide for the laboratory use of zebrafish (*Danio rerio*). Eugene: University of Oregon Press; 2007. Paperback
- Wood FH, Foot MA. Decontamination of potato tubers grown in soil infested with potato cyst nematodes. *New Zealand Journal of Experimental Agriculture*. 1977; 5:315–319.

Figure 1.

Pseudocapillaria tomentosa-egg development. A- egg presenting small blastomeres, B- egg presenting large blastomeres, C- egg with elongated extremity of the embryo, D- egg with turned embryo, E-larval formation inside the eggs. Bars = 10 µm.

Figure 2.
Pseudocapillaria tomentosa-egg development. A, B- hatching larvae, C, D- dead eggs. Bars = 10 µm.

Figure 3.

Assays 1 and 2. Prevalence of *Pseudocapillaria tomentosa*-larvated eggs after treatment following treatment by heating at 28, 40, 50 and 60°C for 30 min. Eggs examined at 1, 3, 5, 7 and 10 post-treatment.

Figure 4.

Assays 3–5. Prevalence of *Pseudocapillaria tomentosa*-larvated eggs after treatment by heating at 28, 40, 50 and 60°C for 1 h. Eggs examined at 1, 3, 5, 7 and 10 post-treatment.

Figure 5.

Assays 9. Prevalence of *Pseudocapillaria tomentosa*-larvated eggs after treatment with chlorine at 100 or 250 ppm for 10 min. Eggs examined at 1, 3, 5, 7 and 10 post-treatment.

Figure 6.

Assays 6 and 8. Prevalence of *Pseudocapillaria tomentosa*-larvated eggs after treatment with chlorine at 500 or 1,000 ppm for 10 min. Eggs examined at 1, 3, 5, 7 and 10 post-treatment.

Figure 7.
Assays 7. Prevalence of *Pseudocapillaria tomentosa*-larvated eggs after treatment with chlorine at 3,000 ppm for 10 min. Eggs examined at 1, 3, 5, 7 and 10 post-treatment.

Table 1

Percentage of dead eggs after treatment by heating at 40, 50 and 60°C for 30 min and 1 h, at 1, 3, 5, 7 and 10 days after counting.

Treatment	1	3	5	7	10
Control _{30 min}	4.8±8.2 ^{Aab}	14.3±24.7 ^{Aab}	5.5±9.6 ^{Aab}	7.4±12.8 ^{Aab}	6.1±10.5 ^{Aab}
40 _{30 min}	0 ^{Aab}	3.7±6.4 ^{Aab}	0 ^{Aab}	0 ^{Aab}	0 ^{Aab}
50 _{30 min}	2.8±4.8 ^{Aab}	11.1±12.7 ^{Aab}	6.1±5.4 ^{Aab}	9.3±1.6 ^{Aab}	26.8±17.4 ^{Aab}
60 _{30 min}	0 ^{Ab}	37.5±54.5 ^{Aab}	29.2±26.0 ^{Aab}	30.6±27.8 ^{Aab}	42.3±25.8 ^{Aa}
Control _{1 h}	0 ^{Ad}	0 ^{Ad}	4.1±3.6 ^{Acd}	0 ^{Bd}	0 ^{Bd}
40 _{1 h}	0 ^{Ad}	0 ^{Ad}	2.8±4.8 ^{Ad}	0 ^{Bd}	0.9±1.6 ^{Bd}
50 _{1 h}	0.9±1.6 ^{Ad}	2.9±5.1 ^{Ad}	1.4±2.4 ^{Ad}	20.1±7.4 ^{Aabc}	24.2±3.3 ^{Aab}
60 _{1 h}	0 ^{Ad}	7.5±13.0 ^{Acd}	9.9±6.2 ^{Abcd}	25.3±13.5 ^{Aab}	29.0±5.7 ^{Aa}
Control _{1 h}	0 ^{Ac}	0 ^{Ac}	0 ^{Ac}	0 ^{Ac}	0 ^{Cc}
40 _{1 h}	2.6±4.4 ^{Ac}	2.8±4.8 ^{Ac}	0 ^{Ac}	5.5±9.6 ^{Ac}	8.9±7.8 ^{Cc}
50 _{1 h}	0 ^{Ac}	3.7±6.4 ^{Ac}	12.1±7.1 ^{Abc}	0 ^{Ac}	29.2±14.4 ^{Bb}
60 _{1 h}	0 ^{Ac}	0 ^{Ac}	4.2±7.2 ^{Ac}	0 ^{Ac}	51.1±15.4 ^{Aa}
Control _{1 h}	0 ^{Acd}	0 ^{Acd}	0 ^{Acd}	0 ^{Bcd}	0 ^{Bcd}
40 _{1 h}	9.8±2.5 ^{Abcd}	3.9±3.5 ^{Acd}	0 ^{Acd}	8.1±9.6 ^{ABbcd}	8.1±9.6 ^{Bbcd}
50 _{1 h}	7.2±7.7 ^{Acd}	3.5±3.3 ^{Acd}	6.5±1.5 ^{Acd}	26.2±8.6 ^{ABbcd}	26.7±7.7 ^{ABabcd}
60 _{1 h}	0 ^{Ad}	8.0±6.9 ^{Acd}	26.7±23.3 ^{Aab}	47.7±31.5 ^{Aab}	50.1±29.2 ^{Aa}
Control _{1 h}	0 ^{Ac}	0 ^{Ac}	0 ^{Ac}	1.4±1.2 ^{Ac}	5.1±3.0 ^{Cc}
40 _{1 h}	0 ^{Ac}	0.9±1.5 ^{Ac}	1.3±1.1 ^{Ac}	0.5±0.8 ^{Ac}	10.7±12.0 ^{BCbc}
50 _{1 h}	0 ^{Ac}	0.6±1.0 ^{Ac}	8.3±1.1 ^{Ac}	4.4±3.9 ^{Ac}	33.8±10.1 ^{ABab}
60 _{1 h}	0 ^{Ac}	0 ^{Ac}	3.5±3.7 ^{Ac}	4.4±1.6 ^{Ac}	56.8±27.7 ^{Aa}

Lowercase letters indicate significant difference among the treatments in the same counting day and uppercase letter indicate significant difference among counting days in the same treatment ($P < 0.05$).

Table 2

Percentage of embryonated (intact but not fully developed) eggs after treatment by heating at 40, 50 and 60°C for 30 min and 1 h, at 1, 3, 5, 7 and 10 days after counting in five separate assays.

Treatment	1	3	5	7	10
Control _{30 min}	90.5±16.5 ^{Aub}	16.7±28.9 ^{Ade}	22.2±25.4 ^{Bde}	14.8±25.7 ^{Bde}	18.2±31.5 ^{Ade}
40 _{30 min}	92.6±12.8 ^{Aa}	63.0±6.4 ^{Abcde}	3.3±5.8 ^{Be}	31.9±6.4 ^{ABcde}	22.2±4.8 ^{Acde}
50 _{30 min}	97.2±4.8 ^{Aa}	76.4±14.6 ^{Abcd}	86.9±16.5 ^{Aab}	82.5±13.7 ^{Aabc}	54.3±19.4 ^{ABcde}
60 _{30 min}	100 ^{Aa}	62.5±54.5 ^{Abcde}	60.0±17.3 ^{ABbcde}	43.1±10.7 ^{ABAbcde}	27.0±15.6 ^{Abcde}
Control _{1 h}	100 ^{Aa}	84.8±3.5 ^{Abcde}	63.1±14.6 ^{Abcde}	49.7±32.6 ^{Abcde}	52.3±35.1 ^{Abcde}
40 _{1 h}	100 ^{Aa}	95.5±7.7 ^{Aabc}	88.9±19.2 ^{Aabcd}	79.0±18.9 ^{Abcde}	72.4±21.9 ^{Abcde}
50 _{1 h}	99.1±1.6 ^{Ab}	85.3±5.7 ^{Abcd}	67.9±13.8 ^{Abcde}	32.4±5.9 ^{Afg}	36.1±10.6 ^{Aefg}
60 _{1 h}	100 ^{Aa}	80.0±12.3 ^{Abef}	69.2±9.0 ^{Abcde}	46.4±25.8 ^{Acde}	40.9±7.0 ^{Ad}
Control _{1 h}	100 ^{ABabc}	100 ^{ABabc}	24.8±13.5 ^{Bf}	27.8±25.4 ^{Bef}	31.9±18.8 ^{Adef}
40 _{1 h}	97.4±4.4 ^{Ab}	93.0±6.4 ^{Ab}	70.8±26.0 ^{ABbcde}	52.2±29.1 ^{ABcde}	52.6±19.4 ^{ABcde}
50 _{1 h}	100 ^{ABabc}	76.8±10.5 ^{ABcde}	70.4±10.6 ^{ABbcde}	83.6±5.6 ^{Aabcd}	70.8±14.4 ^{ABbcde}
60 _{1 h}	95.2±8.2 ^{Be}	86.7±23.1 ^{Bc}	80.5±17.3 ^{Abcde}	77.9±23.1 ^{ABbcde}	48.9±15.4 ^{ABbcde}
Control _{1 h}	97.0±2.7 ^{Aa}	95.1±3.0 ^{Aa}	94.2±1.9 ^{Aa}	91.3±5.9 ^{Aabc}	90.8±6.5 ^{Aabc}
40 _{1 h}	90.2±2.5 ^{Abc}	94.2±2.9 ^{Aa}	92.3±3.4 ^{Aab}	84.9±3.8 ^{Aabc}	84.9±3.8 ^{Aabc}
50 _{1 h}	86.5±1.7 ^{Abc}	86.7±3.0 ^{Abc}	67.3±21.6 ^{Abc}	71.0±4.2 ^{Aabc}	70.3±3.2 ^{Aabc}
60 _{1 h}	95.2±8.2 ^{Aa}	80.2±10.5 ^{Aab}	68.9±23.3 ^{Aabc}	52.2±31.5 ^{Abc}	49.9±29.2 ^{Ac}
Control _{1 h}	100 ^A	99.3±1.1 ^{Aa}	98.9±1.9 ^{Aa}	96.3±2.8 ^{Aa}	81.5±8.2 ^{Aabc}
40 _{1 h}	100 ^A	99.1±1.5 ^{Aa}	96.1±0.7 ^{Aa}	92.8±8.2 ^{Aab}	66.4±4.0 ^{Abc}
50 _{1 h}	100 ^A	97.4±2.2 ^{Aa}	90.2±9.9 ^{Aab}	94.9±2.8 ^{Aab}	54.1±11.8 ^{ABcd}
60 _{1 h}	100 ^A	96.5±3.2 ^{Aa}	93.7±2.7 ^{Aab}	95.5±1.6 ^{Aa}	36.7±32.1 ^{Bd}

Lowercase letters indicate significant difference among the treatments in the same counting day and uppercase letter indicate significant difference among counting days in the same treatment (P<0.05).

Table 3

Percentage of dead eggs after treatment with 100, 250, 500, 1000 and 3000 ppm chlorine for 10 min, at 1, 3, 5, 7 and 10 days after counting.

Treatment	1	3	5	7	10
Control	0.9±1.1 ^{Ac}	0.9±1.1 ^{Ac}	18.5±19.4 ^{Abc}	40.6±40.1 ^{Abc}	71.2±35.8 ^{Aa}
100	2.3±4.7 ^{Ae}	4.3±8.6 ^{Abc}	19.4±36.5 ^{Abc}	26.7±37.8 ^{Abc}	32.5±37.9 ^{ABbc}
250	0 ^{Ae}	0 ^{Ae}	18.2±10.1 ^{Aac}	25.5±9.9 ^{Aabc}	65.9±18.1 ^{Bb}
Control	0.3±0.6 ^{Ae}	1.8±2.8 ^{Ae}	9.0±8.5 ^{Acde}	16.1±17.3 ^{Bcde}	44.3±38.3 ^{Abcd}
500	6.7±5.1 ^{Ade}	7.8±5.4 ^{Acde}	39.0±34.0 ^{Abcde}	37.3±31.9 ^{ABbcde}	43.7±11.2 ^{Abcde}
1000	4.2±6.3 ^{Ade}	21.2±26.8 ^{Acde}	58.5±25.7 ^{Abc}	84.7±10.3 ^{Ab}	91.5±7.0 ^{Aa}
Control	0 ^{Ad}	0 ^{Ad}	1.1±1.3 ^{Ad}	2.5±2.5 ^{Ad}	70.0±17.3 ^{Aa}
500	6.3±9.2 ^{Ad}	3.7±1.6 ^{Ad}	7.1±1.4 ^{Ad}	18.5±7.7 ^{Abcd}	33.0±16.0 ^{Bb}
1000	5.2±3.5 ^{Ad}	2.0±2.4 ^{Ad}	2.4±2.2 ^{Ad}	12.8±5.4 ^{Acde}	30.2±6.8 ^{Bbc}
Control	1.8±2.3 ^{Bb}	1.5±2.0 ^{Bb}	2.0±1.5 ^{Bb}	1.7±93.6 ^{Bb}	42.4±39.7 ^{Bc}
3000	76.1±12.4 ^{Aa}	83.3±5.5 ^{Aa}	94.4±4.3 ^{Aa}	93.6±4.5 ^{Aa}	100 ^{Aa}

Lowercase letters indicate significant difference among the treatments in the same counting day and uppercase letter indicate significant difference among counting days in the same treatment ($P<0.05$).

Table 4

Percentage of embryonated (intact but not developed) eggs after treatment with 100, 250, 500, 1000 and 3000 ppm chlorine for 10 min, at 1, 3, 5, 7 and 10 days after counting.

Treatment	1	3	5	7	10
Control	98.6±1.8 ^{Aa}	96.9±2.3 ^{Aa}	69.2±17.2 ^{Aabc}	44.4±30.6 ^{Acde}	20.1±32.7 ^{Ade}
100	96.4±6.0 ^{Aa}	94.1±7.7 ^{Aab}	46.7±19.2 ^{Acde}	46.2±25.4 ^{Acde}	26.7±19.2 ^{Acde}
250	100 ^{Aa}	99.2±1.5 ^{Aa}	51.2±9.0 ^{Abcd}	40.5±4.2 ^{Acde}	9.2±16.1 ^{Ae}
Control	100 ^{Aa}	100 ^{Aa}	94.2±4.2 ^{Aa}	93.1±5.4 ^{Aa}	29.2±18.2 ^{Abc}
500	93.7±9.2 ^{Aa}	86.8±5.0 ^{Aa}	24.3±7.1 ^{Bbcd}	23.5±9.6 ^{Bbcd}	13.6±3.5 ^{ABcd}
1000	94.7±3.5 ^{Aa}	90.6±3.4 ^{Aa}	31.8±11.5 ^{Bbc}	40.4±9.3 ^{Bb}	7.9±1.7 ^{Bd}
Control	99.7±0.6 ^{Aa}	98.2±3.0 ^{Aa}	91.0±8.5 ^{Aa}	83.9±17.3 ^{Aabc}	49.4±29.7 ^{Abcde}
500	93.2±5.0 ^{Aa}	90.1±1.8 ^{Aab}	43.9±26.8 ^{Bcdef}	36.4±17.8 ^{Bdef}	21.0±7.5 ^{ABef}
1000	95.8±6.3 ^{Aa}	76.1±29.4 ^{Abcd}	35.5±23.9 ^{Bef}	9.8±10.1 ^{Bef}	4.2±6.0 ^{Bf}
Control	98.2±2.2 ^{Aa}	89.7±4.7 ^{Aab}	45.6±31.7 ^{Accl}	57.2±22.8 ^{Abc}	14.6±17.1 ^{Ade}
3000	23.9±12.4 ^{Bcde}	16.7±5.5 ^{Bde}	5.6±4.3 ^{Be}	6.4±4.3 ^{Be}	0 ^{Ae}

Lowercase letters indicate significant difference among the treatments in the same counting day and uppercase letter indicate significant difference among counting days in the same treatment ($P<0.05$).